Novel eco-friendly antimicrobial coatings for use in healthcare and sport textiles

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Abstract

Due to an increase in consumer awareness on environmental and health problems that can arise with synthetic materials and processes used within the textile industry there is a need for novel ‘green’ textiles. The rise in antibiotic-resistant microorganisms within recent years has led to 30,000 deaths in the EU every year, this has led to an increased need for novel antimicrobials (Cassini, Alessano et al., 2016). Synthetic biocides like triclosan and silver have been extensively used in the textile industry but new regulations by the EU Directive 98/8/EC, have now enforced the elimination and withdrawal of many commonly used biocides which are toxic and harmful to humans and the environment (Gao and Cranston, 2008; Gouveia, 2010; Kramer et al., 2006). Plant derived antimicrobials like essential oils (EOs) are therefore an attractive eco-friendly alternative for use in textile finishing (Alihosseini, 2016). Although EOs have regained popularity in recent years, with many studies dedicated to the antimicrobial potentials, (especially citrus-based EOs), few have been focused on their use in blends and encapsulation methods for their application on textiles. Due to EOs physicochemical properties, their development of functional fabrics is met with formulation challenges such as volatility and oxidative degradation and must therefore be protected before they can be used functionally. Screening of ten EOs by disk diffusion and subsequent evaluation of minimum inhibitory concentrations (MICs) and fractional inhibitory concentrations (FICs) showed that a 1:2 blend of *L. cubeba* (litsea) and *Citrus Limon* (lemon) EO respectively had the most efficacy in synergy, being inhibitory against *Staphylococcus aureus*,

Escherichia coli, *Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Trichophyton rubrum* compared to the individual EOs. The litsea-lemon EO blend was encapsulated (30% concentration) with natural biopolymers chitosan (0.05-1% w/v) and sodium alginate (0.1 % w/v) by using an emulsification method, without the presence of a surfactant. Gas Chromatography-Mass Spectroscopy (GC-MS) analysis revealed citral and limonene to be the major compounds found in the EOs, their presence also confirmed by Fourier Transform Infrared (FTIR) analysis. *In vitro,* the release of citral and limonene from the emulsion was examined using a dissolution method and the release profiles were characterised by initial burst release, followed by a slow controlled release of citral and limonene from emulsions; 70.11% of limonene was released within 10 min for a 1% w/v chitosan emulsion, whilst only 4.91% of citral was released within the same time. Fresh 1% chitosan-EO blend emulsions were then used to treat cotton and polyester using a soak-pad-dry method. Promising results were observed when time-kill assays were carried out on the treated fabric using the plate count method adapted from BS EN ISO 20743:2013, with 100% reductions observed at zero contact time (CT) for *S. epidermidis*, at 5 min for *S. aureus* and *E. coli.* Mosquito repellency was also assessed for EO-emulsion treated cotton which demonstrated 71.43% repellency to female mosquito *Aedes aegypti* compared to a repellency of 52.94% by neat EO-impregnated cotton. EOs show promise in their application as antimicrobials for the development of natural and eco-friendly functional textiles and should be further explored as alternatives to current synesthetic based finishing.

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Publications

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- *Novel green antimicrobials for healthcare and sportwear arenas.* Research Degree Students' Poster Competition – De Montfort University – 6 April 2017 – poster presentation

- *Antimicrobial activity of Litsea cubeba, Rosmarinus officinalis and Citrus Limon essential oils against five skin-infection related pathogens.* Postgraduate Poster Competition, Royal Society of Biology (East Midlands branch) – University of Lincoln – 2 May 2017 – poster presentation

- *Antimicrobial activity of essential oils against the growth of healthcare and sportwear infection-related pathogens.* ASM Microbe 2017, New Orleans (USA) – June 1-5, 2017 – poster presentation

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List of Abbreviations

AD	Atopic Dermatitis
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
ATR	Attenuated Total Reflectance
BHI	Brain Heart Infusion
CFU	Colony Forming Units
CH – ME	Chitosan-coated Microemulsions
CI	Creaming Index
CIT	Citral
CMC	Carboxymethyl Cellulose
CS	Chitosan
CT	Contact Time
DMSO	Dimethyl Sulfoxide
DNV	Dilution Neutralisation Validation
DSC	Differential Scanning Calorimetry
DLS	Dynamic Light Scattering
EE	Encapsulation Efficiency
EEA	European Economic Area
EO	Essential Oil
EPS	Exopolymeric Substances
ESBL	Extended-Spectrum Beta – Lactamase
EU	European Union

FC	Final Concentration
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography – Mass Spectroscopy
HAIs	Health Acquired Infections
HCAIs	Healthcare Associated Infections
HEC	Hydroxyethyl Cellulose
HPC	Hydropropyl Cellulose
HPMC	Hydropropyl Methylcellulose
ICU	Intensive Care Unit
IR	Infrared Radiation
LIM	Limonene
LPS	Lipopolysaccharide
LPU	Liquid Pickup
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistant
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MKT	Minimum Killing Time
MRSA	Methicillin–Resistant *Staphylococcus aureus*
MRSE	Methicillin–Resistant *Staphylococcus epidermidis*
MSSA	Methicillin–Susceptible *Staphylococcus aureus*
O/W	Oil–in–Water
O/W/O	Oil–in–Water–in–Oil
OD	Optical Density

PCM	Phase Change Materials
PE	Polyester
PMMA	Polymethyl–Methacrylate
PT	Post Treatment
QAC	Quaternary Ammonium Compound
RT	Retention Time
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SDW	Sterile Distilled Water
SSI	Surgical Site Infections
SSTIs	Skin and Soft Tissue Infections
TGA	Thermogravimetric Analysis
TPP	Sodium Tripolyphosphate
UTIs	Urinary Tract Infections
VRE	Vancomycin Resistant *Enterococcus*
W/O	Water–in–Oil
W/O/W	Water–in–Oil–in–Water

Chapter 1. Introduction

The skin is the natural barrier of the body, protecting us from the external environment and microorganisms that want to invade it (Brodell and Rosenthal, 2008; Venus et al., 2011). When this barrier is compromised by wounds, burns or ulcers, organisms can penetrate into the skin and soft tissues, invading and sometimes causing an infection (Dryden, 2009). Skin infections such as surgical site infections (SSIs) are an extremely common cause of death and morbidity, affecting 6 million people around the world and they are the third most common type of hospital acquired infection (Dryden, 2010; PHE, 2015a; Ranzato et al., 2011). Hospital acquired infections (HAIs) have been estimated to affect 300,000 patients in England with an annual cost of £1 billion to the NHS (Jenkins, 2017). Within the European Union and European Economic Area (EU/EEA), around 2,600,000 new cases of healthcare-associated infections (HCAIs) are reported every year, 426,000 of which have been linked to antimicrobial resistant microorganisms. Antimicrobial resistance (AMR) has been attributed to over 30,000 deaths in the EU per annum (Cassini, Alessandro et al., 2019; Cassini et al., 2016). Since 2002, in the US, 1.7 million cases of HCAI have been reported yearly, with up to 20% of associated organisms being multidrug resistant (MDR) to common antibiotics (Haque et al., 2018; Klevens et al., 2007). Results from epidemiological studies have shown that there is a relationship between overuse of antibiotics and the emergence of antibiotic resistant bacteria (Ventola, 2015). Resistance can be a spontaneous process through the process of mutation or can be caused by gene transfer between bacterial species (Read and Woods,

2014)**.** Wrong prescription of antibiotics has also been deemed as a contributor of antibiotic resistance, with a study showing that in 30-50% of cases, the antibiotic prescribed, and the duration of therapy are incorrect (CDC, 2013; Luyt et al., 2014). Multidrug resistance is worldwide problem which requires new strategies and solutions, one of which is the need for novel antimicrobials; this has led to an increased interest in natural products such as plant essential oils (EOs). EOs have shown promise in inhibiting and preventing the growth of Gram-positive and Gram-negative bacteria, fungi and dermatophytes (Behmanesh et al., 2015; Cavaleiro et al., 2006; Grierson and Afolayan, 2005; Orchard et al., 2017; Rath and Mohapatra, 2015; Zhang et al., 2016).

Many synthetic biocides used to confer antimicrobial activity within the textile industry, such as quaternary ammonium compounds (QACs), silver and triclosan, have been reported to have impressive bactericidal activity however, many also are toxic to the environment, have led to resistance and can cause skin irritation (Gao and Cranston, 2008; Gouveia, 2010; Kramer et al., 2006). The few reported incidences of biotoxicity and low cost associated with natural products such as EOs render them an attractive eco-friendly alternative to synthetic antimicrobials for use in textile product (Alihosseini, 2016). Additionally, due to their complex composition, there is also a reduced expectation for microbial organisms to develop resistance to EOs (Becerril et al., 2012).

Chapter 2. Literature Review

2.1 Healthcare-associated Infections

Healthcare-associated infections are described as those acquired in a healthcare setting such as a hospital (usually presenting after 48-72 hours) or those that develop after a treatment such as surgery, including infections which occur after discharge (4 weeks) from a healthcare facility (Hensley and Monson, 2015; NHS, 2016; WHO, 2016). HCAIs affect millions of people worldwide each year, worsening morbidity and mortality, and financially effecting healthcare systems such as the NHS, as the management of these infections may require further antimicrobial treatment or surgery resulting in longer inpatient stays (Hensley and Monson, 2015; Jenkins, 2017; NHS, 2016; WHO, 2016). In England, it is estimated that 300,000 patients are affected by HCAI, costing the NHS around £1 billion per annum (Jenkins, 2017). In the EU and EEA, over 2 million patients are estimated to acquire HCAI yearly, costing healthcare systems around €7 billion annually, whilst in the US, it is estimated that they incur a yearly cost of $9.8 billion (Babayani et al., 2018; Cassini et al., 2016; Hay, 2005; Judith, 2005; Mei et al., 2015; Porche, 2006). Infections of the urinary tract (27%), lower respiratory tract (24%), surgical sites (17%) and blood stream (10.5%) were the main HCAI observed in Europe in 2008, 19.3% represent other HCAI such as gastrointestinal infections and skin and soft tissue infections.

HCAI are commonly associated with resistant healthcare-associated pathogens such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), Methicillin-Susceptible *S. aureus* (MSSA), *Clostridium difficile*, *Escherichia coli*, and *Pseudomonas aeruginosa* (ECDC, 2008; Hensley and Monson, 2015; PHE, 2015b). Infection numbers of hospital acquired bacteraemia caused by *S. aureus* in England is rising; a total of 11,405 cases have been reported to the PHE in 2015 and 2016, this represents an increase of 7.1% compared to 2014/2015 (n=10,645) and an increase of 15.4% compared to 2011/2012 reports (n=9,883). The incidence of MSSA bacteraemia has increased over time, whilst that of MRSA bacteraemia has fallen, although MRSA is still significant and difficult to eradicate; in 2003/2004, 40% of all bacteraemia related to *S. aureus* was caused by MRSA but only 7.2% of *S. aureus* bacteraemia in 2015/2016 is due to MRSA (PHE, 2016). This, however, is based on data which is limited to one setting (the hospital) and MRSA burden outside a healthcare setting has not been considered (Brusselaers et al., 2011). Decline in MRSA isolation rates have been attributed to infection control measures (e.g. hand hygiene, personal protective equipment and patient care equipment, environmental cleaning and waste disposal) and changes in specific MRSA strains such as ST22 and ST36 which have respectively increased and decreased between 2006-2010 (WHO, 2006; Wyllie et al., 2011)**.**

Primary focus and priority have been and still are currently associated with MRSA, which may be a contributor to the rise in MSSA bacteraemia incidences. Cases of bacteraemia caused by *E. coli* are also increasing, with the

rate of cases per 100,000 population increasing from 60.4 in 2012 to 70.1 in 2015/2016. HCAI caused by *C. difficile* have precipitated over the last years (74.5% since 2007), although this rate of decline has been slowing down, with only a decrease of 0.4% (n=14,192) in 2016 (PHE, 2016). MDR, particularly in Intensive Care Units (ICUs), is considered a significant threat to public health all over the world and is thought to incur additional costs per patient of about $6,000 - $30,000 per annum (Brusselaers et al., 2011). Up to 1.7 million HCAI cases were reported in the US since 2002 with 16-20% of associated organisms found to be MDR, and as the pipeline for novel antibiotics for the treatment of MDR infections is drying out, new strategies and solution must be developed to combat the rising problem (Haque et al., 2018; Klevens et al., 2007).

SSIs are the third most common type of HAI and most SSIs are associated with *Staphylococcus* species, although this notion is being challenged as a study found that on average 6 different genera could be found in any SSI, including staphylococci and *Pseudomonas* spp. (Abboud et al., 2014; Dryden, 2010; PHE, 2015). The number of reported cases of inpatient SSIs caused by *S. aureus* have decreased from 39% to 13% since 2006/2007, which can be contributed to the decrease in MRSA SSIs, which now account for 3% of cases. The number of reported cases of MSSA SSIs have seen very little change in the last years and still account for 10% of SSIs; MSSA is the leading causative organism for hip and knee prosthesis SSIs, neck femur repair SSIs, coronary artery by-pass graft SSIs and spinal SSIs. MRSA, however, is still of concern as a study found that patients with MRSA SSIs were more likely to die within 3 months compared with patients

with MSSA infections (Nelson et al., 2015). Skin infections such as SSIs are the most commonly encountered infections and affect around 6 million people worldwide, making them one of the major causes of death and morbidity around the world (Dryden, 2010; Ranzato et al., 2011).

2.2 The Skin and Infection

The skin is made of two major layers called the epidermis (the uppermost layer) and dermis. The epidermis is then further subdivided into four structurally and functionally diverse layers: the stratum corneum (outer most layer), stratum granulosum, stratum spinosum and the stratum basale. Underneath is the dermis, a tough and resilient structure bound to the epidermis both internally and externally; it becomes very permeable once the epidermis is removed e.g. due to injury. The skin covers the entire body and acts as a barrier to protect us from our external environment; it is described as the “first line of defence against invading microorganisms” (Brodell and Rosenthal, 2008; Venus et al., 2011). The barrier function of the skin is mostly dependent on the stratum corneum and when this barrier is disrupted by a break in the skin such as a burn, wound or ulcer, then a range of organisms can penetrate and colonise the skin and soft tissues and sometimes progress into an infection (Dryden, 2009).

The normal skin of a healthy human-being is usually colonised with a mixture of microorganisms which reside on the skin – this is referred to as the skin flora or microbiota (Dryden, 2010; Egawa and Kabashima, 2016; Gulati and

Nobile, 2016). The type of organism found in the flora of a healthy host will vary based on site, moisture, pH, temperature and salt and lipid levels among others; *Staphylococcus* species and fungal yeast species such as *Candida* are commonly present on the skin as normal commensals (Dryden, 2009; Egawa and Kabashima, 2016; Hensley and Monson, 2015). Other organisms, such as cutaneous mycoses can invade and cause disease in the skin, such as dermatophytic fungi (dermatophytes), which are keratinophilic, having high affinity for the keratin found in tissues of the epidermis, hair and nails (Babayani et al., 2018; Hay, 2005; Judith, 2005; Mei et al., 2015; Porche, 2006).

Table 2.1 Skin structure, functions and infections

Skin layer	Structure	Function	Common infections	References
Stratum corneum	Made of keratin filaments and corneocytes – flattened cells that have lost their nuclei and cytoplasmic organelles.	Acts as a barrier. Main role is protection – regulates entry of particles (microbes, chemicals etc.) and loss of salt and water	**Impetigo** – a contagious pyogenic infection caused by *S. aureus* **Folliculitis** – an inflammation of the hair follicles. Infectious causes can be bacterial e.g. *S. aureus* (barber's itch); or fungal (Tinea barbae). **Tinea capitis** (scalp ring worm), **Tinea pedis** (athlete's foot) – fungal infections caused by *Trichophyton tonsurans* and *Trichophyton rubrum* respectively	(Bhagavatula and Powell, 2011; Dryden, 2009; Dryden, 2010; Ramsay and Török, 2017)
Stratum granulosum	Cells in the layer (keratinocytes) contain intracellular granules of keratohyalin. Cytoplasm contains lamellated granules.	Adhesion, cytokine production, keratin production, production of vitamin D		
Stratum spinosum	Made of a layer of polyhedral cells connected by desmosomes	Responsible for skin's strength and flexibility.		
Stratum basale	Layer is one cell thick made of keratinocytes and melanocytes present.	Responsible for replication and repair of cells		
Dermis	Made of fibroblast cells which synthesise collagen (75% type I and 15% type III collagen) and elastin fibres	Shock absorption (providing protection from injury), Insulation, strength	**Ecthyma** – a contagious infection, with similarities to impetigo but penetrating deeper into the dermis, usually caused by group A streptococci **Cellulitis** – caused by *S. aureus* or group A streptococci is an inflammation of the deep dermis which causes swollen, tender and red skin.	(Ki and Rotstein, 2007; Rabionet et al., 2016; Wilson, M., 2005)

2.2.1 *Wounds, Skin and Soft Tissue Infections*

Health complications can arise during a wound healing process, which delays wounds healing resulting in them becoming severely infected (Sienkiewicz et al., 2017; Song et al., 2016). This delay in healing can occur when microorganism overcome the skin barriers and infect deeper tissues by increasing bacterial load, therefore, causing further damage to the wound and hindering the healing process (Abdel-Mohsen et al., 2016; Said et al., 2014). Biofilms can especially delay healing and their presence in infected wounds can increase mortality, morbidity and treatment cost of infected patients (Song et al., 2016).

Skin and soft tissue infections (SSTIs) are common among hospitalised patients; they occur when microbes invade the skin and underlying soft tissues causing an inflammatory response. SSTIs vary in severity, ranging from mild to life-threatening. SSTIs are hard to diagnose as they can hide behind other clinical symptoms and, due to the increases of bacterial resistance, they are becoming increasingly challenging to treat (Esposito et al., 2016; Ki and Rotstein, 2007). Esposito et al (2016), classified soft tissue infections into two groups, superficial and deep; superficial SSTIs caused by *S. aureus* include impetigo, folliculitis, carbuncles and abscesses, whilst deep SSTIs included cellulitis, myositis, ulcers and wound infections such as skin burns. Complicated SSTIs represent an important clinical and economic burden to the NHS; they often result in

hospitalisation with the need for antibiotic treatment and at times, surgery (Esposito et al., 2016; Lipsky et al., 2014).

The development of measures that will reduce the bacterial load in chronic wounds, as well as new antimicrobial preparations, could be an effective strategy to control chronic wound infections. One of the methods employed to treat wounds is the use of wound dressings that have antibacterial agents, such as antibiotics. Many antibiotics are available, but resistance poses a real threat to wound healing and highlighting the need for developing novel wound care systems (Anjum et al., 2016).

2.2.1.1 Staphylococcus aureus

S. aureus is a Gram-positive, facultative anaerobe that can be found (often harmlessly) in the throat, nose, on mucous membranes and on the human skin in areas such as the groin and the axillae (Agha, 2012). *S. aureus* is a leading cause of nosocomial (hospital acquired) infections; it is able to cause a vast range of infections, such as skin infections, urinary tract infections (UTIs), SSIs, pneumonia, gastroenteritis, and bacteraemia (Ghidey et al., 2014; Hensley and Monson, 2015; Nelson et al., 2015; Wiese et al., 2013). Due to its ability to rapidly develop resistance to antimicrobial drugs, *S. aureus* infections are difficult to treat (Ghidey et al., 2014). MRSA has highly contributed to in-hospital mortality over the years; its resistance to penicillin is facilitated by the production of penicillinase, the enzyme that breaks down part of the penicillin molecule

(Chakraborty et al., 2012). MRSA systemic infections have been linked to mortality rates of up to 40% both in ICUs and non-intensive care settings (He et al., 2013; McDanel et al., 2014; Nelson et al., 2015).

Hospital patients are most susceptible to MRSA infections due to surgical procedures, weaker immune system and indwelling devices (McDanel et al., 2014). Frequently found on healthcare surfaces where it easily lives and proliferates, *S. aureus* has been estimated to be linked to 9000 yearly deaths connected to healthcare contamination (Nan et al., 2015). Not only does *S. aureus* thrive on healthcare surfaces, it also begins to form biofilms, by attaching to the surface and then forming layered cell clusters by a process called intercellular adhesion; once formed, the biofilm is able to give the bacterial strain a greater resistance to antibacterial agents due to the exopolymeric substances (EPS) which make up 90% of the biofilm (Nan et al., 2015; Vickery et al., 2012). Biofilms constitute a problem in places such as healthcare settings where they are thought to be responsible for about 65% of the healthcare acquired infections (Otter et al., 2015). *S. aureus* is commonly associated with wound infections being one of the most commonly isolated bacterial species from chronic wounds, and it has been found to have resistance against vancomycin, erythromycin, quinolones and tetracyclines (Cardona and Wilson, 2015; Gade and Qazi, 2013; Gardete and Tomasz, 2014; Piątkowska et al., 2012). A study by Condò reported antibiofilm activity by cinnamon EO against mature biofilms of Gram-negatives *E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumoniae*, though the biofilms were not completely destroyed (Condò et al., 2018).

2.2.1.2 Staphylococcus epidermidis

Staphylococcus epidermidis is an opportunistic non-motile coagulase-negative, Gram-positive cocci that is part of the normal human skin commensal flora and is in fact is one of the most predominant species found on the skin and superficial mucosal membranes (El Farran et al., 2013; Gomes et al., 2012; Gordon et al., 2012; Hellmark et al., 2009; Hellmark et al., 2013; Lai et al., 2010; Otto, 2012). *S. epidermidis* has become one of the leading pathogens found on devices such as catheters, artificial heart valves and prosthetic joints because of its ability to form a biofilm over the surfaces of these devices, causing infections that are persistent or recurrent (El Farran et al., 2013; Gomes al., 2012; Prasad et al., 2012). *S. epidermidis* was previously considered to be a harmless commensal microorganism but is now regarded as an opportunistic pathogen due to its high incidence in hospitals and its resistance to antibiotics such as methicillin and antimicrobials like benzalkonium chloride (Otto, 2009). Methicillin-Resistant *S. epidermidis*, (MRSE), has been found in 70% of hospital isolates (Gordon et al., 2012) and its intermediate resistance to vancomycin has been on the rise (Otto, 2012). *S. epidermidis* is one of the most prevalent bacteria found on the skin, therefore, when it is isolated from skin infections like wounds, it is usually not considered a pathogen, but dismissed as a contaminant; however, it has been found that *S. epidermidis* could be a carrier of antibiotic resistant genes that can enhance the effect of other pathogens like *S. aureus* (Brackman et al., 2013; Otto, 2009; Otto, 2012).

2.2.1.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic Gram-negative non-fermentative rod that is found widely in humid environments and is sometimes found as part of the human microflora (Loveday et al., 2014). The pathogen is found in hospital settings and can cause a large range of infections such as infected wounds, respiratory, gastrointestinal infections, UTIs and bacteraemia (Kayabas et al., 2008). Reports on bacteraemia in the UK show that *Pseudomonas* spp. are one of the most frequent causes of the disease, and between 2017 and 2018, 37.8% of 4,286 *P. aeruginosa* bacteraemia reported cases were hospital-onset. *P. aeruginosa* is a major cause of infections in immunocompromised cystic fibrosis patients, causing chronic lung infections due to its ability to initiate infection in individuals whose immune system is weak (Breidenstein et al., 2011; Nichols et al., 2013).

Cutaneous injuries such as wounds and burns are often worsened due to colonisation by *P. aeruginosa*, causing infections; *P. aeruginosa* accounts for over 50% of all severe burn infections and is the second most common organisms isolated from chronic wounds like diabetic ulcers, pressure ulcers and in burn wounds (Nichols et al., 2013). With the emergence of multidrug resistant *P. aeruginosa* infections, burn wounds infected with *P. aeruginosa* are becoming difficult to treat. *P. aeuruginosa* is also intrinsically resistant to various antimicrobials such as the common antibiotics ampicillin and amoxicillin (Bessa et al., 2015; Nichols et al., 2013). This microorganism has therefore been

associated with high morbidity and mortality within patients in ICUs, burn units and surgery wards (Kayabas et al., 2008). *P. aeruginosa* can grow successfully in the environment, even in nutrient poor circumstances and over a wide range of temperatures (Loveday et al., 2014). Its ability to thrive and exist in the form of a biofilm on surfaces has contributed to its extreme resistance to several antimicrobials (Breidenstein et al., 2011; Kerr and Snelling, 2009; Knezevic et al., 2013).

2.2.1.4 Escherichia coli

Escherichia coli is a Gram-negative bacillus that is part of the Enterobacteriaceae family (Meybeck et al., 2008). *E. coli* is often associated with UTIs, bacteraemia, soft tissue infections, SSIs and community acquired infections (Ferjani et al., 2015; Niranjan and Malini, 2014; Soto et al., 2006). Strains of *E. coli* are frequently isolated from soft tissue and skin infections; a study found that *E. coli* is the third most isolated bacterium (Petkovšek et al., 2009). It is one of the most common causes of hospital-acquired (over 30%) infections (Smanthong et al., 2015). A study by D'avignon et al (2010) found that, of 3751 patients admitted into a burn unit, 13.8% of deaths associated to bacterial infection were attributable to *E. coli*. A different case study found that within 41 cases of surgical wound infection, 27.1% were related to *E. coli* (Alonso-Isa et al., 2017).

The widespread use of antibiotics for the treatment of infection has led to resistance by organisms such as *E. coli*; a study found that of 161 *E. coli* isolates from wound infection more than 50% were resistant to the antibiotics tetracycline, ampicillin, cefuroxime, ciprofloxacin, cefazolin, mezlocillin and piperacillin. Additionally, 70% of the isolates produced extended-spectrum beta-lactamase (ESBL), an enzyme which causes resistances to antibiotics (Alharbi et al., 2018). Resistance of *E. coli* to antimicrobials has also been linked biofilm formation, as the microorganism is less susceptible to the antimicrobial compared to the planktonic form (Rivardo et al., 2010). Some strains of *E. coli* are highly resistant to penicillin antibiotics (e.g. ampicillin), with a study on the antimicrobial resistance of *E. coli* reporting 82.79% of tested *E. coli* isolates to show resistance to ampicillin (Tompkins, 2011; Vranic and Uzunovic, 2016).

2.2.2 *Trichophyton rubrum*

The dermatophyte *Trichophyton rubrum* is the most prevalent cause of skin (tinea pedis, tinea corporis) and nail (onychomycosis) infections worldwide and accounts for about 70% of all dermatophytoses in human beings (Hammer et al., 2012; Osborne et al., 2003; Vena et al., 2012). Dermatophytes are filamentous fungi, which can infect tissues in which keratin is abundant, such as hair, nail and skin (Baltazar et al., 2012; Mohammadi et al., 2015; Morton et al., 2014). Dermatophytoses (infections caused by dermatophytes) affect millions of people every year and is transmitted by close contact with those infected; it is recognised as a significant public health problem with children in particular

(Bajpai et al., 2009; Feuilhade, 2012; Mohammadi et al., 2015). Dermatophytes like *T. rubrum* are not usually considered opportunistic pathogens, but they occasionally invade soft tissues and organs causing aggressive infections, especially in the immunocompromised (Hay, 2017).

Antibiotics prescribed for *T. rubrum* dermatophytoses (antimycotics) usually include terbinafine, itraconazole, amorolfine and ciclopirox; however, following the completion of antifungal therapy, relapses can occur due to acquired resistance to the antimycotics (Ghelardi et al., 2014; Mukherjee et al., 2003). A study by Ghelardi et al. (2014) observed that *T. rubrum* developed resistance to terbinafine, itraconazole and amorolfine, and concluded that sub-inhibitory drug concentrations can aid in the rise of drug resistant strains (Mukherjee et al., 2003).

2.2.2.1 Tinea Pedis

Tinea pedis (or athlete's foot) is a common and contagious superficial fungal infection (Feuilhade, 2012; Hay, 2013); it is caused by dermatophytes and it is the second most common skin condition in the U.S. and it has been estimated that around 15% of the population suffers from tinea pedis which has significant economic consequences (Ghannoum et al., 2010; Hammer et al., 2012)**.** Tinea pedis usually manifests between the toes (interdigital tinea pedis), on the soles, heels and sides of feet and although not life threatening, the diseases can cause discomfort, may be hard to treat and could spread to other parts of the body

(Zatcoff et al., 2008). Interdigital tinea pedis is the most common form and can make the web spaces between the feet dry, scally, white, macerated and soggy, giving the unpleasant (Zatcoff et al., 2008) sensation of itching, burning or pain and slight odours (Porche, 2006).

Close skin to skin contact among athletes, allows transmission of the fungus from person to person and hyper perspiration, the composition of sweat, temperature and humidity inside of shoes promotes the growth of microorganisms such as *T. rubrum.* Most athletic shoes are made of synthetic materials that poorly absorb water leaving the foot skin exposed to moisture, microorganisms and their by-products for extended durations (Decker, 2010). Textiles that come in contact with infected skin can then be carriers for the propagation of the fungus; contaminated clothing such as socks, shoes, towels and bed linen can therefore be a source of *T. rubrum* infection (Gnanasundaram et al., 2013). It is therefore important that foot insoles in athletic footwear are made of absorbent materials and are also able to fight against microbial growth with the aid of an incorporated antimicrobial agents. Topical antifungals such as imidazoles are usually necessary, with treatment times ranging from 1-4 weeks (Hay, 2005). Some of the most potent topical antifungals are able to destroy the fungi without adverse reactions, but they have drawbacks in that they can be difficult to use, daily application is required as medication is easily removed from the skin through bathing and natural dermal cell turn over (Ikeda et al., 2013).

Few studies have focused on alternative or natural treatment of tinea pedis. Treatment of patients with athlete's foot using copper-oxide impregnated socks over a 9-day period, has shown promising results, with all 56 patients showing improvement or resolution of erythema, vesicular eruptions, scaling and itching associated with the disease (Zatcoff et al., 2008). However, studies have shown that dermatophytes are susceptible to the action of (plant derived antimicrobials) and therefore more investigations need to be dedicated to the treatment of tinea pedis using natural agents (Bajpai et al., 2009; Pereira et al., 2011).

2.2.3 *Eczema*

Eczema or atopic dermatitis (AD), is one of the most common inflammatory skin diseases in the general population and affects approximately 15-20% of children and 10% of adults in the UK. In up to 50% of patients AD will improve before adolescence, whilst for others which present AD until adulthood, the disease is chronic and recurrent (Archer, 2013; de la Fuente-Núñez et al., 2013; Orfali et al., 2013). Patients with AD, are more to susceptible to infection or colonization by various organisms including *S. aureus; which* is consistently found in patients (80-100%) with AD skin lesions and therefore, plays a significant role in the exacerbation of AD (Barnes and Greive, 2013; Boguniewicz and Leung, 2013; Gong et al., 2006). AD is often treated with topical or systemic antibiotics, however, the emergence of antibiotic resistance and concerns about antibiotic abuse, has posed a challenge for treating AD infections. The use of

bleach baths (sodium hypochlorite) in the treatment of AD is being explored but the safety and efficacy still needs to be proven by further clinical trials and alternatives are still being sought (Barnes and Greive, 2013). AD can have a significant impact on the quality of life of the families involved; sleep loss, absence from school and pruritus (itching) discomfort are often consequences of AD. Atopic dermatitis is a chronic disease that has no cure and requires effective preventative measures (de la Fuente-Núñez et al., 2013).

2.2.4 *Wound Management*

While most wounds heal without complications, there are some chronic and acute wounds in which the healing process is delayed, leading to increase in patient mortality and leaving serious scars (Singh et al., 2017). Wound management forms a critical step in the care of patients, and this is especially true with surgery patients. The possibility of wound complications, such as SSI, means that the at the basic level, a dressing should provide an environment that is clean, provide a barrier to protect the patient from microbial contamination, absorb any extensive exudate, reduce pain and the risk of scarring and may also actively intervene in the healing process (Koehler et al., 2018; Vowden and Vowden, 2017). Factors such as stress, nutrition, immunity and patient health or lack thereof all contribute to wound healing and they must all be managed together to achieve optimal wound healing. Due to bacterial activity, wound healing is also accompanied by odour production, most especially in patients with chronic wounds (Vowden and Vowden, 2017).

With the aim of improving wound healing, various types of wound dressings have been developed with both natural and synthetic materials, such as membranes, hydrogels, films, hydrocolloids and sponges, however not all are suitable for every type of wound (Simões et al., 2018). For example, hydrogels are mechanically weak when swollen (due to water absorption) and often require a second dressing but are able to keep a moist environment around the wound due to their ability to store water within a polymeric network (Kamoun et al., 2017; Koehler et al., 2018). Sponges are also possess good ability to keep wounds moist during healing due to their high porosity but are not suitable for the protection of third-degree burns and may cause softening and breaking down of the skin tissue due to the prolonged exposure to moisture (Ramos-e-Silva and Ribeiro de Castro, 2002). Films used as wound dressings provide adequate protection from contamination and bacteria but can be hard to manage and may not effectively prevent exudate build up (Kamoun et al., 2017). Membranes also provide good protection, acting as barriers between the wounds and the environment and ensuring normal gas exchange and cell production can take place, however, the materials (such as polymers) and solvents used in producing the membranes may cause issues with biocompatibility (Bhattarai et al., 2004).

As such, natural polymers are being used in the production of bioactive wound dressing; alginate based wound dressings (such as sodium alginate) have been explored and have become commercially available (e.g. Algisite™). Alginate dressings are characterised by a high water (or exudate) uptake and

sustained drug release which promote rapid wound healing (Aderibigbe and Buyana, 2018; Boateng et al., 2008; Dhivya et al., 2015). It has been reported that alginate-based dressings are able to either initiate or accelerate the healing of wounds due to the activation of macrophage and the production of TNFalpha protein which aids in the resistance to infection (Thomas et al., 2000).

2.2.4.1 Antimicrobial Wound Dressings

Due to the high rates of morbidity and mortality attributed to wound infections there has been an increase in the development of wound dressings which incorporate antimicrobials with the aim of preventing microbial contamination in wounds (Simões et al., 2018; Woodford and Livermore, 2009).

Silver biomaterials have been successfully used in the process of wound healing with several silver-containing products having been developed for use in dressings. Silver nanoparticles are thought to be able to interact with bacteria cell structures, such as the cell wall; they are able to penetrate the cell wall and cause structural changes which affect the cell membrane permeability and cause cell death (McShan et al., 2014). Another mode of action considered is cell membrane damage caused by contact with free radicals formed by the nanoparticles (Prabhu and Poulose, 2012). Their broad antimicrobial activity against MRSA, vancomycin-resistant *enterococci* (VRE), yeasts and moulds have been the driving force behind research in silver-based products. However, tissue irritation (due to non-specific binding of silver ions) and delayed healing of

burn wounds (especially silver sulfadiazine) are some of the disadvantages faced with silver wound dressings (Ashtikar and Wacker, 2018; Atiyeh et al., 2007). Additionally, the effectiveness of silver-based wound dressings has been challenged by Wang et al (2007) as a study on their use on chronic wounds found that treatment duration for patients using silver-dressings was greater (p>0.0001) compared to those using other dressings (Wang et al., 2007). The emergence of resistant strains of *E. coli* after prolonged use of silver-based dressings also means that new products need to be developed which will have less harmful effects on humans (Ashtikar and Wacker, 2018; Atiyeh et al., 2007).

Natural antimicrobials are therefore being researched; manuka honey wound dressings are being used for the management MRSA infected wounds, with a clinical study by Visavadia et al (2008) observing healing of MRSA infected wounds within 4 weeks of treatment without complications (Visavadia et al., 2008).

2.3 Essential Oils

Plants naturally protect themselves against microbes and pests by producing secondary metabolites such EOs, which are products of this secondary metabolism of aromatic plants (Yap et al., 2013). EOs are produced within the organs of aromatic plants, such as flowers (rose, violet, litsea, lavender, jasmine), herbs (mint), buds (clove), leaves (thyme, salvia, eucalyptus), zest (citrus), and

roots (ginger). Due to their hydrophobic nature and lower density (compared to water), EOs are immiscible with water but are soluble in organic solvents such as alcohols and in the presence of surfactants (Asbahani et al., 2015). EOs are usually obtained from hydrodistillation, steam distillation and solvent extraction and they are comprised of a complex mixture several individual compounds (Okoh et al., 2010).

EOs are known for their characteristic strong fragrances and for their antimicrobial (fungicidal, bactericidal and virucidal) and anti-inflammatory properties (Asbahani et al., 2015; Bakkali et al., 2008; Calo et al., 2015; Pandey et al., 2014; Raut and Karuppayil, 2014). EOs have been increasingly studied for their antimicrobial efficacy since the 1990s, in fields such as food, dentistry, agriculture, disinfection and pharmaceutics and their use is increasing in industry due to consumer demand for more natural ingredients based on health and environmental awareness (Asbahani et al., 2015; Carvalho et al., 2015; Fisher and Phillips, 2006; Khani and Asghari, 2012; Pattnaik et al., 1996; Quintas et al., 2015; Vital et al., 2016; Yangui et al., 2009).

2.3.1 *Antimicrobial Activity of EOs*

Due to the increasing prevalence of multi-drug resistant pathogenic microorganisms, there is a need for more effective antimicrobials that have different modes of action; this has led to an increased interest in natural products antimicrobial activity (Pandey et al., 2014; Wong et al., 2014).

It has been found that not only are EOs able to effectively kill bacteria in the planktonic phase, but they are also able to eradicate bacteria within biofilms (Kavanaugh and Ribbeck, 2012). EOs are naturally occurring antimicrobials and their activity is directly correlated to the presence of bioactive compounds such as alcohols, terpene compounds, esters, ketones amines, sulphides and aldehydes; these are grouped into terpene compounds and aromatic (or phenolic) compounds (Calo et al., 2015; Patel and Gogna, 2015). Though, these compounds usually only represent a small fraction of the plant composition (Asbahani et al., 2015). Antimicrobial sensitivity is for EOs can be measured using various methods such as the diffusion method (either disc diffusion or agar diffusion), the dilution method (either by microdilution using 96-well plater or by macrodilution using test tubes) and the time-kill method (Orchard and van Vuuren, 2017).

Some EOs have shown promising antibacterial activity against nosocomial Gram-positive bacteria; cinnamon oil, for example, has antimicrobial efficacy against *S. aureus*, with a MIC of 1.0 mg/ml and MBC of 2.0 mg/ml., cinnamaldehyde (92.40%) was thought to be the main component responsible for this antimicrobial activity (Zhang et al., 2016). The content of cinnamaldehyde is much higher in the study by Zhang et al (2016) compared to studies by Li, Kong and Hong (2013) and Li et al (2011) where the compositions were 81.97% and 77.35% respectively (Li et al., 2013; Lv et al., 2011; Tian et al., 2016). A study on oregano EO showed activity against *S. aureus* although MICs observed in the

dilution method were high (>256 mg/ml) indicating low sensitivity to the EO (Alexopoulos et al., 2011). *S. aureus* has also been found to be susceptible to lemon grass EO (zones of inhibition >8.50 cm) also inhibiting biofilm formation at 0.125% v/v (Adukwu et al., 2012). Carvacrol an antimicrobial phenol that often occurs in EOs has also been reported to have biofilm activity against *S. epidermidis* (MIC value not reported) biofilm formation and established biofilms, with a great reduction in biomass (>50%) though activity was only studied at acidic pH (Nostro et al., 2012).

Cinnamon EO was found to have antimicrobial against nosocomial Gram-negatives, including those that are antibiotic-resistant including carbapenem-resistant *P. aeruginosa* and *A. baumannii isolates;* with the highest zones of inhibition observed being 4mm and 35mm respectively. Only one isolate of *P. aeruginosa* was found to be resistant to cinnamon EO, when compared to CLSI standard break points. Results showed promise; however, no MIC or MBC values were determined (Kaskatepe et al., 2016). Gram-negatives such as *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens*, have shown susceptibility to *Salvia namaensis* (Namibian sage) EO with low MIC values of 1.24, 1.25 and 0.63 mg ml^{-1} respectively (Grierson and Afolayan, 2005). *P. aeruginosa* has demonstrated resistance to some EO; in a study by Demo et al (2005), 14 plants EOs were analysed against *P. aeruginosa* and other organism, and none of the oils tested were able to inhibit *P. aeruginosa* (Demo et al., 2005). Cinnamon and coriander (*Coriandrum sativum)* EOs have also been found to be antibacterial against *E. coli* with a study by Zhang et al (2016) reporting at an MIC of 1.0 mg/ml

and an MBC of 4.0 mg/ml for cinnamon EO and another study by Bazargani and Rohloff (2016) reporting an MIC value of 1.6 µl/ml for coriander (Bazargani and Rohloff, 2016; Zhang et al., 2016).

Studies conducted on EOs, against fungi and dermatophytes have also demonstrated promising antimicrobial efficacy. Lavender EO has showed antifungal activity against *C. albicans* demonstrating similar efficacy to antifungal agent clotrimazole (Behmanesh et al., 2015). However, *Ocimum basilicum* (basil), *Rosmarinus officinalis* (rosemary) and *Salvia officinalis* (sage) EOs were found to not have any antifungal activity against *Candida* isolates at a range of concentrations (50 – 3200 µg/ml). *Nigella sativa* (black cumin), *Murraya koienigii* (curry leaf), *Piper betel* (betel lead) and *Trachiyspirum ammi* (ajwain) EOs were all found to be antimicrobial against *Candida* strains, even after a heat treatment of the EOs, with MIC values ranging from 15.62 – 250µl/ml; of the four EOs, ajwain and black cumin leaf showed the greatest antifungal activity with MIC values as low as 31.25 µl and 15.62 µl respectively. Ajwain EO also showed the fastest Minimum Killing Time (MKT) against *C. albicans*, with a kill time of 10 mins at 37°C and at room temperature. Interestingly, the MKT is reduced when the test is carried out at lower temperatures (4°C), and instant kill was observed at this temperature against *C. albicans* (Rath and Mohapatra, 2015). This could be due to volatility of major antimicrobial compounds at higher temperatures which could have an adverse effect on antimicrobial activity of the EOs; Turek and Stintzing (2012) reported characteristic changes and degradation of EO component (α-terpinene) in rosemary EO when stored at 38°C (Turek and Stintzing, 2012). A

reduction in biofilm adherence of *C. albicans* has been also been observed in the presence of coriander EO at concentration below MIC 15.6μg/ml (Freires et al., 2014). *Juniperus* (juniper) EOs were evaluated against fungi, including *Candida* strains, *Aspergillus* strains and the dermatophytes *T. rubrum* and *T. mentagrophytes*. Both dermatophytes were found to be susceptible to the antimicrobial activity of all juniper EOs, with EOs of juniper leaves (*Juniperus oxycedrus)* being the most active, giving an MIC and Minimum Lethal Concentrations (MLC) values of 0.08-0.16 μl/ml for *T. rubrum* and *T. mentagrophytes*. In comparison, Juniper leaves EO showed very little activity against *Aspergillus* strains compared to activity against dermatophytes, with MIC and MLC values between 1.25 – 5.0 μl/ml (Cavaleiro et al., 2006).

The composition of EOs is thought to be responsible for the antimicrobial activity observed in the various studies carried out in literature and their composition can easily change during storage and processing due to the degradative effects of light, temperature and oxygen on the compounds found in EOs (Nguyen, H. et al., 2009). The volatility and susceptibility to degradation by the environmental of these individual compounds means that the EOs need to be protected if their antimicrobial properties are to be used effectively; one of the most effective ways of protecting EOs is through encapsulation.

2.4 Encapsulation of EOs

Encapsulation technology has become a useful method in pharmaceutical, textile, fragrance, agricultural and food industries. It provides protection for the core material against degradation, allows a controlled release of the active agent and can lower the loss or extend the flavour of food over time (Fernandes et al., 2014; Haidong et al., 2012; Martins et al., 2014). EOs comprise compounds that are prone to react with light (photosensitive), oxygen and are sensitive to high temperatures; such reactions can lead to degradation and therefore reduce the activity of EOs. Thus, there is a need to encapsulate EOs to prevent such reactions; encapsulating EOs can also increase the stability of the volatile compounds during heating process (Dima et al., 2016).

In the last decade; there has been an increased use of natural biodegradable polymers, within various industries; polysaccharides in particular have gained interest due to their favourable properties in terms of safety (FDA approval), toxicity, biocompatibility, availability and cost (Liu et al., 2015; Yang et al., 2015). Polysaccharides with biological activity (such as gum Arabic, chitosan and sodium alginate) have been of interest in recent years, for novel biomedical applications such as wound healing, drug delivery and tissue engineering (de Barros Fernandes et al., 2014; Hsieh et al., 2006; Kanakdande et al., 2007; Pedro et al., 2009). In addition, the ability to control their molecular weight and their chemical composition to achieve the microcapsule properties desired make them a favourable choice (Gong et al., 2014). Microencapsulation allows the protection of a substance allowing easier handling for application; the system allows large amounts of lipophilic substances to be loaded within the lipid core and the shell

membrane to protect the core ingredients from unfavourable environmental conditions during processing or storage and can control the release rate of the core material through the wall membrane (Butstraen and Salaün, 2014; Lv et al., 2014; Nakagawa and Nagao, 2012).

2.4.1 *Microencapsulation Technology*

In simple terms, a microcapsule is comprised of a core (usually the active component needing protection) and wall materials (also referred to as the coating or shell) which are commonly polymers, carbohydrates or proteins (Bakry et al., 2016; Haidong et al., 2012). Methods of encapsulating the active material vary and their use will be depended on the nature of the core material, the desired particle size, desired release of the core and intended the application of the final product (Ghayempour and Montazer, 2016; Haidong et al., 2012). The process of microencapsulation involves the coating of droplets or particles of a substance (e.g. drugs, hormones, proteins, fertilizers, cosmetics, oils) with a thin wall of natural or synthetic polymers that acts as a protective barrier, to create individual particles (Butstraen and Salaün, 2014).

There are various methods of microencapsulation including physical (e.g. spray-drying and freeze-drying), chemical (e.g. solvent evaporation and *in situ* polymerization) and physicochemical (e.g. ionic gelation, coacervation and emulsification) methods (Tomaro-Duchesneau et al., 2013).

2.4.1.1 Complex Coacervation

Complex coacervation is based on the coacervation from an oil-in-water (o/w) emulsion, in which the emulsion, which contains an anionic surfactant, is added to an aqueous solution (e.g. of chitosan); after which an electrolyte (e.g. alginate) is added to form microcapsules (Butstraen and Salaün, 2014). Microencapsulation requires the cross-linking of the wall polymers to increase the thermal and mechanical properties of the capsules (Butstraen and Salaün, 2014; Zhang et al., 2012). Most of the cross-linking agents used to create microcapsules are toxic in nature and have to be washed out with a solvent to reach a biologically acceptable level, restricting its use in many applications; such cross-linkers include aldehydes i.e. glutaraldehyde and formaldehyde (Butstraen and Salaün, 2014; Yang et al., 2014; Zhang et al., 2012). Sodium tripolyphosphate (TPP) is non-toxic cross-linker that has been proposed as an alternative to aldehyde crosslinkers (Butstraen and Salaün, 2014).

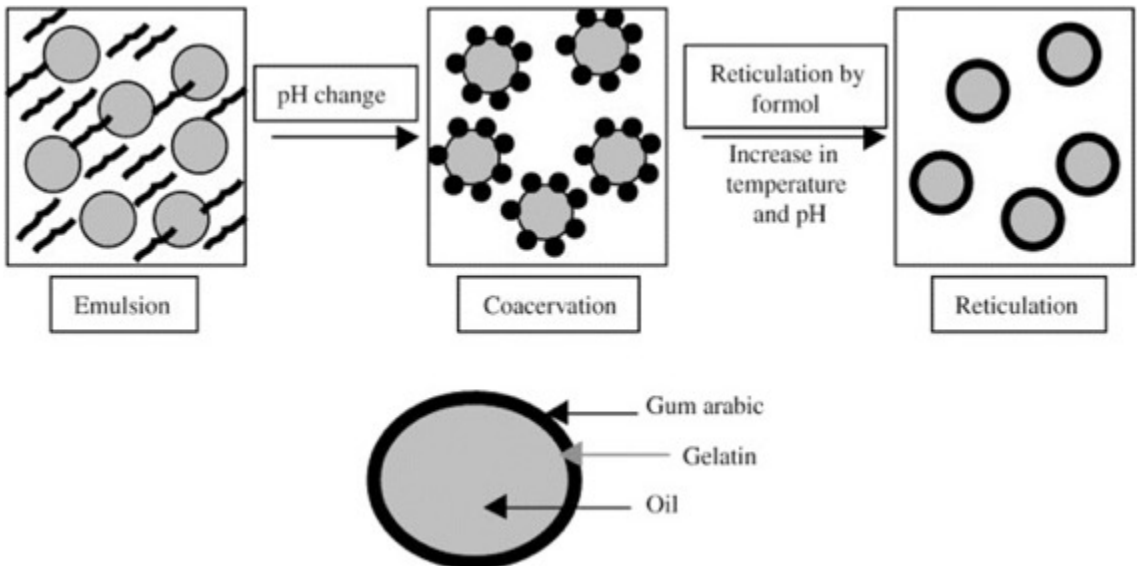


Figure 2.1 Complex coacervation process (Madene et al., 2006).

Complex coacervation, illustrated in Fig 2.1, is achieved under specific conditions that are dependent on the charge and charge density of the wall polymers used, the processing temperature and the process itself, such as cooling and stirring (Piacentini et al., 2013). During complex coacervation a spontaneous reaction occurs between two polymers of opposite charge, leading to a phase separation in which an aqueous phase and a polymer phase are formed once the charges are neutralized (Piacentini et al., 2013). Complex coacervation is a promising technique for the production of micro/nanoparticles within industry; it is simple, allows high payloads, good controlled release and heat resistant properties, high efficiency and does not require a solvent (Lv et al., 2014; Nakagawa and Nagao, 2012; Yang et al., 2015). In a microencapsulation process based on coacervation, the pH is a key parameter. Aziz et al (2014), evaluated the effects of core material (krill oil) to wall material (gelatin-gum Arabic) ratio, stirring speed and pH on the encapsulation efficiency, and found that pH had the most significant effects on the encapsulation efficiency (EE). Stable microcapsules, with 92% EE were synthesised using optimal conditions of pH 3.8, stirring speed 3, and a ratio (of core material to wall material) of 1.75:1 (Aziz et al., 2014). Stirring speed is important because the microcapsules can be significantly affected by the homogenization rate during the process of emulsification; when a lower rate is used during preparation, the microcapsules release the core material more rapidly than those prepared with a higher rate during the process (Zhang et al., 2012). Microcapsules produced by complex coacervation are also affected by the polymer properties including molecular mass, ionic charge density and concentration, all of which are determined by the

formulation (Nakagawa and Nagao, 2012). Microencapsulation of *Melaleuca alternifolia* (tea tree) EO by complex coacervation led to an increase in the evaporation temperature of tea tree EO from 140°C to 230 – 260°C because of the core protection provided by the polymers gelatine (G) and sodium carboxymethyl-cellulose (C). The ratio of these polymers (G/C), affected the formation of the coacervate during synthesis and the EE of tea tree EO. The increase in G/C ratio lead to an increase in EE (63.3 ± 1.4%) up to G/C=10, because of the amount of coacervate formed, and above this value, the amount of oil in the microcapsules decreased again (Pérez-Limiñana et al., 2014). The process, however, is not without its challenges; aggregation and release problems (burst) have been reported, which are not desirable for most of its applications (Yang et al., 2014).

2.4.1.2 Ionic-Gelation

Ionic-gelation is a method that has received a lot of attention specifically in the preparation of chitosan and TPP microcapsules, which are used for the *in vivo* administration of drugs (Fàbregas et al., 2013). The process is non-toxic, convenient, controllable, and does not require the use of organic solvents The method involves the complexation between the positively charged amino groups of a polymer and the oppositely charged groups of a cross linking agent to form a system that is sensitive to ionic strength (Dong et al., 2013; Fàbregas et al., 2013; Fan et al., 2012). By using this method, the use of chemical cross-linkers and emulsifiers is avoided; this is especially useful as most of these chemicals

are usually toxic (Fan et al., 2012). Spray drying, although economical and widely used, uses high temperatures up to 160 °C, which accelerates the oxidation of oils, and requires the combination of different wall materials to minimise lipid oxidation (Abang et al., 2012; Carneiro et al., 2013). The coacervation method, compared to the ionic gelation method, although efficient, is expensive; this method was proposed by Abang et al (2012), however, their study, using inverse gelation produced spherical capsules with diameters around 3 mm, the production of smaller microcapsules or nanocapsules was not demonstrated (Abang et al., 2012). Martins et al (2015) optimised the process by adjusting the experimental conditions (wall material ratio and concentration, curing time, stirring rate) and achieved core-shell microcapsules with a smaller 500μm mean diameter (Martins et al., 2015).

2.4.1.3 Freeze-Drying

Freeze-drying is also known as lyophilization, a method used to dehydrate heat-sensitive substances such as oils; it has been used to encapsulate fish oil and olive oil (Calvo et al., 2012; Heinzelmann et al., 2000). It operates by lowering pressure and freezing the material; the ice is then removed by sublimation as the material transitions directly from a solid phase to gas phase (Krokida and Philippopoulos, 2006). Advantages of freeze-drying include ease of operation, simplicity and protection of heat sensitive materials (Bakry et al., 2016). Velasco et al (2003) found that freeze-drying reduced sensitivity of oils to oxidation but decreased the encapsulation efficiency (Velasco et al., 2003). Freeze drying,

uses high amounts of energy, is high cost and involves a long process. Additionally, the material (e.g. the oil) could be more exposed to the environment when freeze-dried because of the resulting powders are highly porous, though this feature is advantageous when a high drug release is required (Bakry et al., 2016; Sinha et al., 2007).

2.4.1.4 Spray Drying

Spray drying is one of the oldest and most established process of encapsulation and has been used to prepare pharmaceutical products such as granules, suspensions and dry powders. Spray drying involves forming an emulsion by dispersing the core material in a polymer solution; this is then homogenized and atomized into a drying chamber (Ixtaina et al., 2015). The method is commonly used to encapsulate core materials that are sensitive to heat, and functional lipophilic ingredients (Berendsen et al., 2015; Dima et al., 2016; Munoz-Ibanez et al., 2016). A disadvantage of spray drying is that the release properties can be affected due to the formation of amorphous systems, which are thermodynamically unstable and can change back to the crystalline state on storage. Spray drying can also affect particle size; dexamethasone acetate containing PLGA nanoparticles were formulated and spray dried, the spray drying processes significantly ($p< 0.05$) increased the nanoparticle sizes. The mean particle size increased from 200 ± 60 nm to 230 ± 100 nm, however particle density and size distribution seemed to be unaffected (Gómez Gaete et al., 2008).

2.4.1.5 Emulsification

Emulsification is a simple and cheap method used to encapsulate bioactives (such as EOs and extracts) within aqueous solutions. These solutions are then ready to be used in liquid form but can be further processed using the methods described above. Emulsions comprise two phases, usually oil and water, which are immiscible (hydrophilic and hydrophobic); the hydrophobic (oil) phase can be dispersed within an aqueous phase, forming an oil-in-water (O/W) emulsion or the opposite can be true forming a water-in-oil (W/O) emulsion (Bakry et al., 2016). Polymer microcapsules have been created using this method with polymethyl-methacrylate (PMMA) and jasmine EO; spherical PMMA microcapsules, with smooth surfaces were achieved when the PMMA to jasmine EO weight ratio was 2:1 and 3:1. At a 1:1 ratio, the polymer capsules could not be achieved, as the capsules comprised holes, possibly due to insufficient amounts of PMMA required to coat the jasmine oil droplets. Encapsulation efficiency was determined to be 72%, as 4.78mg of jasmine oil was found, when analysing 20 mg of dried capsule (Teeka et al., 2014).

2.4.1.6 Release of the Core Material

The release of the core material from the microcapsule is measured by using gentle agitation to achieve a well-mixed dispersion and then the changes in the solute concentrations over time are measured either in intervals or

continuously. The release kinetics and the diffusion coefficient can therefore be calculated (Gray et al., 2015). The release rate of an active ingredient within a microcapsule depends on different factors including: the wall material, the core material itself, the morphology and geometry of the particle, the degree of cross-linking, the conditions (e.g. pH, temperature, ionic strength) and the method of microencapsulation e.g. spray drying (da Silva et al., 2014; Dima et al., 2016). A study on chitosan-encapsulated menthol microcapsules observed that the crosslinkers used (TPP) had an effect on the release time of menthol when used at different concentrations, and generally the higher the TPP concentration, the slower the release time. At 1% w/w TPP, 95% of menthol was release within 60 h, whilst at 15% w/w TPP, only 38.3% was released at 60 h (Nuisin et al., 2013). It is important that the release of the core material occurs at the appropriate time and place; a study by Nuisin et al (2013) shows that the release rate is mainly related to interactions between the core and wall material (da Silva et al., 2014). The pH of the microcapsule environment can also have an effect on the release rate of the core material; drug containing alginate-pectin microcapsules showed higher drug release percentages in acidic pH 1.2 compared to an alkaline pH 8.2, with maximum drug release of 75.6% and 42% respectively (Jaya et al., 2009). The characteristics of the core material, including amount can also affect release rate; Dürrigl et al (2011) found that their calcium loaded microparticles, showed greater drug release with a higher drug load and a 2:1 drug: polymer ratio (Dürrigl et al., 2011). Release studies are conducted using mathematical models that describe the different ways that molecules are transported across the capsule wall. Larger microcapsules are more suited to controlled release applications

because there is a reduced protection of the core material by the wall material and therefore the release rate of the core material is improved (Dong et al., 2011).

2.4.1.7 Biodegradable Polymer Materials

Natural polymers are divided into polysaccharides, nucleic acids, proteins and lipids (Yang et al., 2015). Polysaccharides have attracted a lot of recognition as promising wall materials for encapsulation, due to their 'superior physicochemical and biological properties; they are safe, non-toxic, biodegradable, biocompatible, are abundant in nature, can be modified easily and low cost (Amin et al., 2014; Liu et al., 2015; Yang et al., 2015). They are made of long chains of monosaccharide units linked by glycosidic bonds; included on their chains can be reactive groups such as hydroxyl, carboxyl, and amino groups on their chains, which are reactive, and allow the molecules to be modified (Amin et al., 2014; Liu et al., 2015; Yang et al., 2015). Chitosan is a renewable natural marine-derived polysaccharide that is extremely abundant (Banerjee et al., 2002; Rodrigues, Susana et al., 2012; Yang et al., 2015). Chitosan is already being used within the pharmaceutical industry because it is biocompatible, biodegradable, non-toxic and has bio adhesive and antibacterial properties gained through the alkaline deacetylation of chitin (Avadi et al., 2010; Banerjee et al., 2002; Butstraen and Salaün, 2014; Dima et al., 2016; Dong et al., 2013; Du et al., 2009; Qi et al., 2004; Trapani et al., 2009). Alginate is another natural and abundant polysaccharide (Pawar and Edgar, 2012). Alginates are used in industry based on their viscosifying, stabilizing and gelling properties; they are

used as stabilizers and thickeners within the food industry; medically, alginates have been popular in wound care (as wound dressings), as alginate dressings can provide an acceptably moist environment for the wounds exudate whilst absorbing great amount of secretions (Draget and Taylor, 2011; Goh et al., 2012). Cellulose is a biopolymer found abundantly in vascular plants as well as in algae, and some bacterial species (Kumar and Turner, 2015). It is the most widely used, most abundant, renewable, biodegradable, and biocompatible polymer, and its derivatives include carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC), hydropropyl-cellulose (HPC) and hydropropylmethyl cellulose (HPMC); all have been recognised for the biocompatibility (Amin et al., 2014; Song and Chen, 2015).

2.4.2 *Characterisation of Microcapsules*

2.4.2.1 Particle Size and Size Distribution

Microcapsules often come in different sizes and size distributions depending on the methods used and their size are related to their mechanical properties e.g. smaller microcapsules will have lower rupture force compared to a larger microcapsule. A study by Sun and Zhang (2002) showed that the bursting force and deformation of melamine-formaldehyde, urea-formaldehyde and gelatine microcapsules increased proportionally with their diameter (Sun and Zhang, 2002). To determine the particle size of microcapsules, dynamic light scattering (DLS), laser diffraction and microscopy are used. There are limitations with the use of laser diffraction, as the refractive index of the shell material must

be known for the measurement. Microscopy has an advantage over laser diffraction as it gives the true image of the microcapsule but it time consuming and is therefore, mostly used for individual size analysis rather than size distribution (Gray et al., 2015). DLS a method that gives you the average particle size and the size distribution within the sample elucidating the homogeneity of particles being analysed.

2.4.2.1 Surface Charge

The zeta potential (the charge at the interface between a particle and the medium surrounding it) needs to be controlled to prevent microcapsule aggregation. The zeta potential should be analysed to identify what needs to be done to move the zeta potential away from the isoelectric point in order to prevent aggregation; electrophoresis is one of the methods used to calculate the zeta potential by measuring the electrophoretic mobility of the microcapsules in a medium (Gray et al., 2015). A study on chitosan coated microemulsions (CH-MEs) evaluated the zeta potential of CH-MEs was increased after being coated with chitosan solution but decreased when surfactant concentrations increased; results were then used to develop CH-MEs with optimal stability and acceptable physicochemical behaviour (Kesavan et al., 2013).

2.5 Application of Microcapsules in the Textile Industry

The textile industry has been a major generator of employment and income through the years; in the European Union (EU), 2.45 million people were employed in 2006 within the textile industry. However, it has been deemed one of the most polluting industries in the world, with harmful chemicals being used, high water and energy consumption, large generation of waste, high fuel consumption from transportation and the use of non-recyclable packaging materials have all contributed to the unsustainable life cycle of textiles (Hasanbeigi and Price, 2012; Jena et al., 2015; Parisi et al., 2015). The various stages of textile production generate waste into the environment and require vast amounts of water, energy and chemicals contributing to environmental problems such as water pollution (Jena et al., 2015; Parisi et al., 2015). All stages of the production process of textiles use vast amounts of water, energy, fuel and chemicals, especially the preparation process, dyeing and finishing. A textile mill that produces 8 tonnes of fabric a day will use an average of 1.6 million litres; 16% of this is used for dyeing and 8% for printing (Parisi et al., 2015; Sinclair, 2014). Also, each stage generates waste, which ends up being injected into the environment, effectively polluting it (Jena et al., 2015). More sustainable processes are being investigated to reduce this environmental impact and create more sustainable products Manufacturers are faced with the task of making a sustainable product that is produced with the environment, fair trade and human rights in mind (Parisi et al., 2015). In the recent years, there have been improvements in environmental law enforcement, and increased demand for

sustainable clothing from ethical consumers, and importantly, a greater compliance by manufacturers as the awareness of the need for sustainability increases.

Increased knowledge of the environmental issues associated with the textile industry has led to increased demand for natural-fibre based textiles like cotton, and natural dyes and finishes (Jena et al., 2015; Shahid-ul-Islam et al., 2013). Greater awareness has also led textile processors to try and reduce their environmental impact (Jena et al., 2015). Consumer demand for textiles that offer functional properties, such as insect-repellence, flame retardation, UV protection and antimicrobial protection; such textiles are called 'functional textiles' has also increased, in addition, textiles that provide protection from infection by pathogenic microorganisms have become increasingly sought after (Hui et al., 2013; Shahid-ul-Islam et al., 2013; Specos et al., 2010).

The current focus in the textile industry, involves the microencapsulation of phase change materials (PCM) to produce textiles with an added value, such as medical textiles and thermo-regulating fabric (Sánchez et al., 2010). Consumers are however, becoming increasingly aware that bacteria can grow and survive on fabrics, especially those that are used in environments such as hospitals, contributing to disease transmission, but also contributing fabric deterioration, skin irritation and development of foul smell (Riley et al., 2017). There is an increasing interest in producing antimicrobial finishes using natural sources, such as plant extracts containing active compounds; not only are these

eco-friendly but also are derived from sources that are renewable (Sumithra and Raaja, 2012). There is potential application of natural antibacterial fabrics in arenas such as healthcare and sportswear but only a few published studies have been carried out on the incorporation EOs to textiles to create antimicrobial fabrics. Sayed et al (2017) applied a nanoemulsion encapsulating neem EO on cotton fabric using the padding technique and reported 71.73% and 65.69% reduction of *S. aureus* and *E. coli* after 4 washes (Sayed et al., 2017).

2.5.1 *Antimicrobial Finishes*

Finishes are important in increasing the usage characteristics and the appeal of a textile product (Jena, et al. 2015). Textiles made of natural fibres are a good medium for microbial growth as they provide the conditions and nutrients required for growth. Synthetic fibres, however have been found to be resistant against microbial degradation and discolouration, due to high hydrophobicity; for instance polyester (PE) has a lower moisture absorbance than cotton and therefore, it is more likely that microbial growth will occur on the skin causing odour problems (Dev et al., 2009; El-Ola, 2008). Deterioration of fabric is often linked to microbial growth, therefore antimicrobial finishes are being sought to prevent the growth of microorganisms on textiles, and within medical field, with an added aim of preventing cross-infection by pathogens to patients, healthcare workers and the environment (El-Ola, 2008).

A number of chemical agents have been used to impart antimicrobial activity to textiles, which include antibiotics, nanometals, onium salts, nitro compounds, inorganic salts, phenols and thiophenols, but many of these are toxic to humans and are not easily degradable within the environment; finishing's involving chitosan and EOs are therefore being explored (Fu et al., 2011). Fabrics, like cotton can be treated with natural finishes that would potentially combat hospital-acquired infection like chronic wounds, dermatophyte infections such as athlete's foot and even secondary infections from eczema.

There is evidence that EOs have promise as antimicrobial agents within various fields and industries and an increased interest and demand for antimicrobial finishes, with the textile industry looking for more eco-friendly processes especially those processes that can reduce the chemical burden. To date, there are limited studies on how biopolymers such as chitosan can be used in combination with natural antimicrobials like EOs for the purpose of antimicrobial finishes on fabrics. The principal aim of this thesis is to investigate the possibility of using natural antimicrobials to provide antimicrobial properties to textiles in an eco-friendly manner. The vision, upon fabrication of an effective antimicrobial eco-friendly textile, is that the textile would have potential both in the healthcare and sports arenas against healthcare associated infections and sports related infections.

2.6 Scope of Works

Although increasing evidence has been reported that EOs show potential as antimicrobial agents, there is limited data on the use of blends of citrus EOs, in particular litsea and lemon blends, as an antimicrobial for the application in textile finishing. With an increasing demand for eco-friendly finishes within the textile industry, studies on the use of biopolymers such as chitosan and sodium alginate for the encapsulation of EOs, (with the intention of bestowing antimicrobial properties) are limited.

2.7 Aims and Objectives

The aim of this study is to develop an antimicrobial coating for textiles using a novel green microencapsulation process (with the exclusion of toxic processes and substances) for EOs that could be utilised in the clinical and sports arenas for skin related conditions and malodour.

Objectives:

- To identify effective antimicrobial EOs and EO blends against bacterial strains of *S. aureus, E. coli, S. epidermidis, P. aeruginosa* and the dermatophyte *T. rubrum*;
- To formulate EOs and eco-friendly microcapsule wall materials (biodegradable polymers) into a stable emulsion;

- To apply the formulated emulsion onto textiles using a simple and toxic free process;
- To assess treated fabric for antimicrobial activity and durability.

Chapter 3. Materials and Methods

3.1 Universal Methods

3.1.1 *Microorganisms and Culturing Methods*

The test microorganisms, *S. aureus* (NCTC 8327) isolated from skin, *P. aeruginosa* (NCIMB 8626), *E. coli* (NCTC 8003) isolated from gastro-enteritis and *S. epidermidis* (NCTC 5955) isolated from skin lesions, were obtained from Public Health England, UK. *T. rubrum* (ATCC 28188) isolated from human lesions was obtained from Thermo Scientific, UK.

All bacterial organisms were cultured in Brain Heart Infusion (BHI) broth (53286) and maintained on BHI agar (70138) from Sigma Aldrich (Gillingham, UK). All bacterial organisms were grown under aerobic conditions at 37°C. *T. rubrum* was cultured in Sabouraud dextrose broth (SDB) (CM0147) and maintained on Sabouraud dextrose agar (SDA) (CM0041) from Oxoid (Hampshire, UK). *T. rubrum* was grown under aerobic conditions at 30°C. All cultures were stored on beads at -80°C.

3.1.2 *EOs*

Bergamot (*Citrus bergamia*), citronella (*Cymbopogon nardus*), lemon (*Citrus limon*), litsea (*Litsea cubeba*), bitter orange (*Citrus aurantium var amara*), sweet orange (*Citrus aurantium var sinensis*), peppermint (*Mentha piperita*),

rosemary (*R. officinalis*), rosewood (*Aniba rosaeodora*) and wild thyme (*Thymus serpyllum*) EOs were obtained from Penny Price Aromatherapy Ltd (Hinckley, UK).

3.1.3 *EO Blend*

The combination of the EOs litsea/lemon (1:2 ratio) were prepared before each experiment.

3.1.4 *Growth Curves*

100 ml of BHI broth in conical flasks was inoculated with a colony of either S. *aureus*, *S. epidermidis* or *E. coli* and incubated at 37°C for 24 hours. The optical density of the cultures was recorded every hour at 590 nm using an Evolution 60S UV-Visible spectrophotometer (Thermo Fisher Scientific, UK). Samples of the cultures were taken hourly for 10 hours and then again at 24 hours. Serial dilutions were carried out, aliquots of 0.1 µl were plated and incubated at 37°C for 24 hours before enumeration of viable counts.

T. rubrum was pre-cultured on SDA and plugs were collected from the cultured plates using a metal corer and transferred onto the centre of fresh SDA plates. Plates were incubated at 30°C for 15 days. The radial growth of *T. rubrum* was recorded using a digital calliper every day for 5 days, on day 8, 9, 10 and finally on day 15.

Examples of typical growth curves for *S. aureus, S. epidermidis*, *E. coli* and *T. rubrum* can be seen in Appendix I.

3.1.5 *Chemicals used in Emulsion Preparation and Characterisation*

Authentic standard of analytical grade citral (99%), (R)- limonene and (S)-limonene were obtained from Sigma Aldrich (Gillingham, UK). Polymers of chitosan (low molecular grade) and sodium alginate were obtained by Sigma-Aldrich (Dorset, UK). Calcium chloride was obtained from Fisher Scientific (Loughborough, UK).

3.1.6 *Statistical Analysis*

All statistical analysis was carried out using SPSS version 25 for Windows with significance set at $p = 0.05$ (unless stated otherwise). The Kolmogorov-Smirnov test was used to test assumptions of normality and variances of homogeneity were assessed using Levine's test. If all assumptions were met, data was analysed using one-way analysis of variances (ANOVA). Tukey post-hoc tests were used to determine if there were any significant differences between group means. If any assumptions were violated the Welch test was performed. ANOVA output example can be found in Appendix III. All investigations were carried out in triplicate on at least two separate occasions, unless stated otherwise and data is presented as mean ± standard deviation (SD).

3.2 Screening EOs for Antimicrobial Activity

3.2.1 *Inoculum Preparation*

Bacterial suspensions of *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. epidermidis were* prepared by transferring a colony from a freshly grown plate of bacteria into 10 mL of BHI broth; inoculate broths were then incubated at 37°C for 18 hours. Fungal spore suspensions of *T. rubrum* were prepared by releasing 1ml of sterile distilled water (SDW) containing 0.01% Tween 80 in the centre of a pre-cultured SDA plate and gently scraping the spores with a glass spreader. The spore suspension was then made up to 5ml with SDW and filtered using a ply of 5 sheets of muslin cloth sheets of 5 cm x 5 cm squares to remove the hyphae and spore thread. After filtration, the spores were washed 3 times by centrifuging the suspension at 2000 x *g* for 5 mins at room temperature, supernatant removed, adding 5 ml of SDW and resuspending the spores by mixing with a shaker; this was repeated three times. The spore count was then adjusted using a haemocytometer/nephelometer and microscope to (10^7) CFU/ml with SDW.

3.2.2 *Disc Diffusion Method*

The disc diffusion method was used to assess the antimicrobial activity of all 10 EOs. Aliquots of 50µl of overnight cultures of *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. epidermidis* at a concentration of 10^8 CFU/ml, or of a 10^7 spore suspension of *T. rubrum,* were spread over the surface of BHI agar plates. Filter discs (20 mm) were then placed over the center of the agar plates and

impregnated with 25 µl of EO by directly pipetting onto the filter disc after placement. All plates were incubated at 37°C for 18 hours for bacteria and at 30°C for 7 days for fungi; the ZOIs were then measured and recorded using digital callipers. Controls were plates without oil impregnated filter discs.

3.3 Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of Antimicrobial EOs

Doubling dilutions of EOs with final concentrations between 40 µl/ml and 0.1 µl/ml were prepared for litsea, lemon and rosemary EO in BHI/SD broth, with the addition of 10% v/v DMSO solvent (Fisher Scientific) to make a final volume of 9.9 ml. Aliquots of 100 µl of an overnight culture of either *S. aureus, P. aeruginosa*, *E. coli* or *S. epidermidis* (10^8 CFU/ml) were then added were then transferred into 96 well plates. For fungal organism 100 µl of a fresh spore suspension (10^7 CFU/ml) was transferred to test tubes. Optical density (595 nm) readings were taken at 0 hour and again at 24 hours after incubation of bacterial organisms at 37°C, on a SpectraMax Plus 384 microplate reader and results were analyzed using SoftMax Pro version 6.4 software (Molecular Devices, USA). Readings for the fungal organism were taken visually by checking for visible growth. The MIC for each EO was defined as the lowest concentration of EO that inhibited the visible growth of the microorganism. The MBC was determined by spread plating inoculated EO solutions onto BHI, incubating at 37°C for 24 hrs and enumerating. The same method was carried out for *T. rubrum*, using SDA

and incubating at 30°C for 7 days. Controls were microorganism in the presence of 10% v/v DMSO only.

3.4 Assessment of Synergism between EOs

To investigate the potential of synergistic interactions between the EOs in double and triple combinations, the checkerboard method was used; the fractional inhibitory concentration (FIC) and the FIC Index (or FICI) were then determined for rosemary, lemon and litsea EOs. The EOs were combined in 96 well plates, to yield varying concentrations ranging from several dilutions below the MIC to double the MIC. Serial concentrations were prepared with BHI or SDB broth (for fungi) with 10% (v/v) DMSO emulsifier for bacterial organisms or 10% (v/v) Tween 80 emulsifier for fungi. These were then inoculated with either *S. aureus*, *P. aeruginosa*, *E. coli*, *S. epidermidis* (BHI) or *T. rubrum* (SDB). Double combinations and triple combinations of the different concentrations of EOs were produced and MIC was determined as described above in Section 3.3. The FIC and FICI of each combination of EOs was then calculated for the double and triple concentrations according to Equations (1) and (2) respectively:

$$FIC_{indexA/B} = \frac{MIC\ (oil\ A\ )combined}{MIC\ of\ oil\ A\ alone} + \frac{MIC\ (oil\ B\ )\ combined}{MIC\ oil\ B\ alone} \quad (1)$$

$$FIC_{indexA/B/C} = \frac{MIC\ (oil\ A)combined}{MIC\ of\ oil\ A\ alone} + \frac{MIC\ (oil\ B)\ combined}{MIC\ oil\ B\ alone} + \frac{MIC\ (oil\ C)\ combined}{MIC\ oil\ C\ alone} \quad (2)$$

The synergistic, additive, non-interactive (or indifferent) and antagonistic effects were determined from these limits: FICI ≤ 0.5 indicates a synergistic effect, whilst 0.5 ≤ FICI ≤ 1 indicates an additive effect, 1 ≤ FICI ≤ 4 indicates no interaction and FICI > 4 indicates an antagonistic effect (Hossain et al., 2016; Stein et al., 2015).

3.5 Emulsion Preparation and Microencapsulation

Emulsions of 10% final EO-blend (litsea/lemon) concentration containing 20% v/v Tween 80 surfactant (Fisher Scientific, UK) and emulsions containing 10% EO but no Tween 80 were prepared. Emulsions containing 20% v/v Tween 80 were prepared by mixing the surfactant with 5 ml of litsea-lemon EO blend (1:2 ratio) using a magnetic stirrer. A solution (5 ml) of 0.025% sodium alginate was then added into the oil phase and mixed with gentle stirring at room temperature. Subsequently, 5 mL of calcium chloride solution (18 mM) was

added dropwise into the polymer-oil mixture and mixed for 5 min until a gel was formed. Once the gel was formed, a solution of chitosan in various concentrations (0.05 – 1.5 % w/v in final o/w emulsion) was added dropwise into the gel and the solution was mixed for 45 minutes to form an o/w emulsion. Once formed, the primary emulsion was homogenized (IKA Ultra-Turrax T25 Homogenizer) at 8000 rpm for 1 hour.

Surfactant-free emulsions were prepared by first mixing 5 ml of the EO-blend with 1.25 ml of a 0.025% sodium alginate solution for 5 min using a magnetic stirrer. After which, a solution of chitosan in various concentrations (0.05 – 1.5 % w/v in final o/w emulsion) was then added to the mixture and pre-homogenised for 5 minutes at a speed of 8000 rpm. An 18 mM solution of calcium chloride (5 mL) was then added to the EO-polymer mixture, dropwise, under further homogenisation at 8000 rpm for 1 hour. The o/w emulsions were stored at room temperature for 24 h before being analysed.

Once it was determined the formulations did not display visual instability (separation) without the addition of a surfactant but displayed complete separation with the addition of Tween 80 (Figure 3.1), 50 mL batches were produced with the final concentration of EO in the o/w emulsions of 30% v/v. The amount of sodium alginate used was increased to 5mL of 1% v/v solution and the calcium chloride concentration was also increased by using 5mL of a 1% v/v solution. All emulsions were tested for pH measurements using a FG2-Kit pH Meter (Mettler Toledo, USA) 24 hours after preparation. The pH meter was

calibrated using buffer solutions and pH measurements were carried out on samples at room temperature to ensure pH of emulsions was below 5.

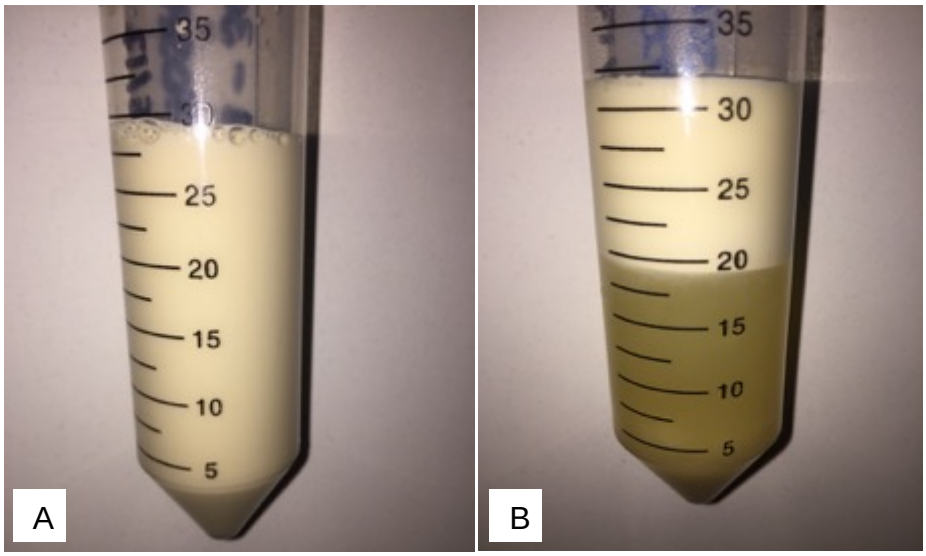


Figure 3.1 Photographs of chitosan-sodium alginate emulsions with 10% litsea-lemon EO blend prepared a) without surfactant b) with 20% Tween 80 surfactant

Table 3.1 Litsea-lemon EO blend o/w emulsions and concentrations of chitosan, sodium alginate and calcium chloride in the water phase used for emulsion preparation.

Batch	EO blend (%v/v)	Sodium Alginate (%w/v)	$CaCl_2$ (%v/v)	Chitosan (%w/v)
F1	30	0.1	0.1	2.00
F2	30	0.1	0.1	1.25
F3	30	0.1	0.1	1.00
F4	30	0.1	0.1	0.75
F5	30	0.1	0.1	0.50
F6	30	0.1	0.1	0.25
F7	30	0.1	0.1	0.125
F8	30	0.1	0.1	0.05

3.6 Antimicrobial Activity of Litsea-Lemon EO O/W Emulsions

Assessment of the antimicrobial activity of the surfactant-free emulsion batches with 30% EO final concentrations (F1-F8) and the activity of the individual emulsion components (1% chitosan solution, litsea-lemon EO blend (1:2 ratio), 1% sodium alginate solution and standards of citral, (R) and (S) limonene) were assessed individually against *S. aureus*, *S. epidermidis* and *E. coli* using the disk diffusion method as described in Section 3.2. For all emulsions tested 20 mm

Whatman filter paper disks (Scientific Laboratory Supplies, UK) were used while 6 mm disks were used for EOs, polymers and standards.

3.7 Identification of Main Compounds in *L.* cubeba and *C. limon* EOs

GC-MS analysis of litsea and lemon EOs was performed using a Bruker 450-GC coupled with a CP-8400 autosampler (Bruker Corporation, USA) and 300-MS SQ signal electron ionisation. Both EOs were diluted (1:100) in *n*-hexane (Fischer Scientific, Loughborough, UK) and filtered through a 0.45 µm polyethylene (PET) filter (Sigma-Aldrich, Gillingham, UK). The injected sample volume was 1 µL with a split ratio of 1:100. The GC was coupled with a Rxi-5ms column (Restek, Pensylvannia, USA) column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The ionizing energy of the MS was -70 eV, with CID gas pressure of 1.5 mTorr and a detector voltage of 1000 V. The injector and transfer temperatures were 280°C and 250°C respectively. Source temperature was at 230°C analysing mass range (50 – 350 M/z). Helium was used as a carried gas at a constant flow rate of 1.5 ml/min. The oven temperature program was 60°C for 5 min, followed by a 4°C /min ramp to 220°C and finally an 11 °C/min ramp to 250°C held for 15 min. EO components citral and limonene (were identified by comparing with retention time and mass of pure standard reference compounds (Sigma-Aldrich, UK). Data was analysed using a Bruker MS workstation software (version 7.0). Calibration curves for citral and limonene compounds between 0.78125 – 200 mmol were created to allow for the quantification of the compounds within the emulsions.

3.8 Rheology

Emulsion rheological behaviour and the viscosity of 1% w/v chitosan and 1% w/v sodium alginate were measured using a Physica MCR 501 Rheometer (Anton Paar GmbH, Austria). A CP25-1 diameter measuring cone with a 25 mm cone angle (Anton Paar, GmbH, Austria) was used to carry out oscillatory rheological measurements at a gap size of 0.047 mm. Shear rate sweep with an up ramp from 0.05 s^{-1} to 50 s^{-1} was carried out at 20 ˚C. Undiluted samples of 0.5-1% CS emulsions were evaluated 24 hr after preparation. Average viscosity is reported as a function of shear rate.

3.9 Zeta Potential

A NanoBrook Omni Zetasizer (Brookhaven Instruments, UK) was used to measure the zeta potential of the emulsions. The emulsions were diluted 1:100 using distilled water prior to analysis to measure the zeta potential of the emulsions (0.5-1% CS) after 24 h of storage at 4°C. Analyses were carried out at 25°C and 8 scans were performed for each sample. A quartz cuvette was used for analysis.

3.10 Particle Size Determination

The particle size of the emulsions and its distribution were determined using a dynamic light scattering (DLS) technology using a NanoBrook Omni

Particle Sizer (Brookhaven Instruments, UK). The emulsions (0.5-1% CS) were diluted in distilled water to 1:100 and the measurements were performed five times (each with 3 runs) at 25°C. The width of distribution, defined by the polydispersity index (PI) was determined according to Equation (3).

$$\mathrm{PI} = \frac{d(90) - d(10)}{d(50)} \quad (3)$$

Where *d*(90), *d*(50) and *d*(10) and) are the particles diameter at 90%, 50% and 10% cumulative volume, respectively (Campelo et al., 2017).

3.11 Optical Microscopy

After 24 h of storage, the structure of the litsea-lemon emulsions (0.5-1% CS) was observed using an optical microscope (DM750 Brightfield Microscope, Leica Microsystems, Germany). The emulsion samples were diluted 1:10 with purified water and the observation was performed at room temperature.

3.12 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) analyses were carried out on a Bruker Alpha FTIR spectrometer (Bruker Corporation, Germany) with an attenuated total reflectance (ATR) platinum diamond sampling stage. The infrared radiation (IR)

analyses were conducted on emulsions, pure chitosan, pure sodium alginate, litsea EO, lemon EO and reference standards of limonene and citral with a scanning range of 400-4000 cm^{-1}. For each spectrum 10 scans at a resolution of 2 cm^{-1} were obtained and spectra were obtained with OPUS software version 7.5 (Bruker Corporation, UK).

3.13 Thermogravimetric Analysis

The thermal properties, stability and decomposition of the prepared emulsions, its individual components (litsea EO, lemon EO, chitosan, sodium alginate and calcium chloride) and standards of citral, (R) and (S) limonene were investigated using a thermogravimetric analysis (TGA) apparatus (PerkinElmer, USA). Samples were analysed using a temperature range of 25 – 400 °C at a heating rate of 10 °C/min in the presence of nitrogen gas as an inert atmosphere.

3.14 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) experiments were carried out using a Jade DSC (PerkinElmer, USA) to analyse the thermal properties, stability and decomposition of the *L. cubeba*/*C. limon* emulsions, it's individual components and reference standards. The aluminium pans containing the solid samples were placed inside the DSC in addition to an empty reference pan. Solid samples were sealed with perforated lids, whilst liquid samples were sealed with non-perforated lids. Samples were first cooled down to -50°C and then heated

back up to 400°C. The heat flow rate used was 20˚C/min in the presence of nitrogen purge gas at a rate of 20 mL/min.

3.15 Release Studies

A dialysis system was used to assess the release of limonene and citral from the litsea-lemon emulsions (0.5 – 1% CS) using a paddle dissolution apparatus. The emulsions (20 mL) were transferred into 43 mm wide cellulose membrane dialysis tubes (14,000 kDa molecular weight cut off, Sigma-Aldrich). The emulsion-loaded (20 mL) dialysis bags were suspended release medium of acetate buffer (pH 5) with 2% v/v Tween 80; temperature and stirring were set at 32°C (to mimic normal skin temperature) and 50 rpm respectively. Aliquots of 5 mL were withdrawn at specific time intervals (0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min), filtered and analysed by GC-MS for the amount of limonene and citral released; theoretical concentrations of limonene and citral are 1.53 mmol/ml and 114.33 mmol/ml respectively. Each sample withdrawn was replaced with 5 ml of fresh acetate buffer of equal temperature. The cumulative percentage of citral and limonene released in the medium was obtained by dividing the cumulative amount of citral and limonene released at each sampling time point $(\mathrm{M_t})$ to the theoretical initial concentration of citral and limonene in the sample $(\mathrm{M_0})$ according to Equation (4).

$$\%\ \text{Cumulative Release} = \sum_{t=0}^{t} \frac{M_t}{M_0} \times 100 \qquad (4)$$

3.16 Stability of Litsea-Lemon EO O/W Emulsions

3.16.1 *Determination of Emulsion Physical Stability*

The creaming stability was assessed by measuring 5 mL of the o/w emulsions into measuring cylinders, storing the cylinders at room temperature as well as observing and recording daily the volume of creaming during storage for 5 weeks. The creaming index (CI) was estimated according to the Equation (5).

$$\% \text{ Creaming Index} = \frac{H_c}{H_t} \times 100 \quad (5)$$

Where H_c is the height of clear layer below the sample and H_t is the total emulsion height. The measurement of creaming index indicates the kinetic formation of a clear layer (separation) caused by droplet aggregation and its increase can provide indirect information on the stability of the emulsion. Emulsions tested were those containing 0.5%, 0.75% and 1% chitosan concentration.

3.16.2 *Determination of Emulsion Long-term Physical Stability*

To predict the long-term stability of the emulsions an accelerated centrifugation test was also carried out to observe the degree of creaming. The centrifugation test was carried out by taking 10 mL samples of emulsions and centrifuging (Rotanta 460s Heltich-Zentifugen) at 4600 rpm for 2 hours. The

emulsions were observed every 5 min for separation and the percentage Creaming Index under stress conditions was characterized using Equation (5). Emulsions samples tested were those containing 0.5%, 0.75% and 1% chitosan concentration. All samples were analysed in triplicate.

3.16.3 *Determination of Emulsion Chemical Stability*

The chemical stability of emulsions of 0.5%, 0.75% and 1% chitosan at 15°C and at 40°C was assessed for 4 weeks and assessed for chemical changes upon storage (only values at time 0 and day 28 are shown). The emulsions were stored in dark temperature regulated cabinet and the changes in concentration of citral within the emulsions after 28 days was assessed by taking 1 ml samples and carrying out solvent extractions using up to 5 mL of *n*-hexane (Fisher Scientific, UK) and by shaking for 5 minutes. Samples (1 mL) from the extraction mixture were filtered through a 0.45 µm PET filter and the filtered extractions were analysed for the presence of citral using the same method described in Section 3.7.

3.17 Treatment of Polyester and Cotton Fabric

Prior to treatment, knitted cotton and knitted polyester fabric samples were first washed using a detergent scouring solution of 2 g/L of non-ionic wetting agent Ultravon PL (Ciba Specialty Chemicals, UK) for 30 min at 60°C, followed by a hot and cold-water rinse. The fabric was then sterilized at 160°C for 2 hours

by dry heat sterilization. Once sterile, the fabric samples were soaked for 15 minutes into fresh emulsions prepared as described in the method in Section 3.5. Each soaked fabric sample was passed through a two-roller laboratory pad (Ernst Benz, Switzerland) at a pressure of 35 kg/cm at a speed of 1 m/min; samples were then re-soaked into the emulsions and passed through the padding machine a second time. The process was repeated for each antimicrobial emulsion (0.5 %, 0.75% and 1% CS). The weights before soaking and after padding were recorded to calculate the percentage liquid pick up (LPU) according to Equation (6):

$$\%\, LPU = \frac{(weight\ of\ wet\ fabric) - (weight\ of\ dry\ fabric)}{(weight\ of\ dry\ fabric)}\ 100 \qquad (6)$$

The fabric samples were then left to dry overnight at room temperature, before being used for antimicrobial testing.

3.18 Quantification of Distribution of Major EO Components on Polyester and Cotton Fabric Treated with 1% CS Emulsion

Polyester and cotton fabric samples were treated as described in Section 3.17 and cut into samples of 4 x 4 cm, 2 x 2 cm and 1 x 1 cm size taken from throughout the textile, each were then weighed to determine the density of the

treated fabric. Solvent extractions were carried out on each sample using 5 mL of *n*-hexane (Fisher Scientific, UK) and by shaking vigorously for 5 minutes. Aliquots of 1 mL from the extraction mixture were filtered through a 0.45 µm PET filter and the filtered extractions were analysed for the presence of citral and limonene using the same method described in Section 3.7. The remaining treated fabric samples were stored at 4°C for 7 days and the extraction process was repeated as above.

3.19 Toxicity Test and Dilution Neutralisation Validation for Treated Fabric

Validation of dilution-neutralisation and toxicity test were adapted from Annex C of the BS EN 1276:2009 standard. A bacterial or fungal working culture (10^7-10^8 CFU/mL) was prepared and the suspension serially diluted (in PBS for bacterial organisms or SDW for the dermatophyte) and enumerated.

Neutralizing solution

- 10 g/L Tryptone
- 5 g/L sodium chloride
- 30 g/L Saponin
- 1 g/L L-histidine
- 30 g/L Tween 80
- 3 g/L Asolectin from soybean
- 5g/L Sodium Thiosulphate

3.19.1 *Toxicity Test*

To ensure that the neutraliser is not toxic to the microorganisms, a neutraliser toxicity test was carried out by adding 1 mL of working culture to 9 mL of neutraliser. The solution was mixed and incubated in a water bath at 20°C for 30 min and then mixed again. After the contact time (CT), the solution was serially diluted, and the plates incubated at 37°C for 18-24 h (bacteria) or 30°C for 7 days (fungi), after which colonies were observed and enumerated. Absence of toxicity was confirmed by comparing the growth of the control and that within the test conditions.

3.19.2 *Dilution Neutralisation Validation*

To ensure the neutraliser is effective against the emulsion treated fabric, the dilution-neutralisation validation (DNV) test was performed by adding 9 mL of neutraliser to 0.40 g fabric samples placed in vials. This was left in contact for 5 min on a water bath at 20°C. After the CT 1 mL of bacterial/fungal working culture was added to the neutraliser mixture and left in contact for 30 min at 20°C in a water bath and mixed at the end of the CT. Serial dilutions were then carried out, plated and incubated at 37°C for 18-24 h for bacteria or 30°C for 7 days for fungi, after which colonies were observed and enumerated. Neutralising efficacy was confirmed by comparing the growth of the control culture and the test conditions.

3.20 Qualitative Determination of Antibacterial and Antifungal Activity of Polyester and Cotton Fabric Treated with 1% CS Emulsion

The antimicrobial and antifungal activity of the treated fabric (100% polyester and 100% cotton) against *E. coli, S. aureus, S. epidermidis, T. rubrum*, *E. coli* (clinical isolate 390685Q), *S. epidermidis* (clinical isolate PLO 21862) and MRSA (antibiotic resistant strain NCTC 12497) was determined using the agar diffusion plate test adapted from BS EN ISO 20645:2004. This qualitative method was used to screen the treated fabric for bacteriostatic activity. Polyester and cotton test samples with diameters of (25 ± 5) mm were cut out of treated and untreated fabric. A lower layer (not inoculated) for each petri dish was prepared by either pouring 10 ml of molten BHI (bacteria) agar or SDA (fungi) in sterile petri dishes, these were then left to congeal. Agar for the upper layer was prepared and cooled to 45 ± 1°C in a water bath and then 150 ml of the molten agar was inoculated with 1 ml of an overnight working culture (10^8 CFU/ml). The inoculated agar was mixed to distribute the bacteria evenly. Aliquots of 5 ml were then poured into each petri dish and left to congeal. Once set the test samples of treated and untreated fabric were pressed onto the surface of the medium with a pair of sterilized forceps to ensure good contact between the fabric sample and the agar. The plates were then incubated for 18-24 h at 37°C for bacteria and for 7 days at 30°C for fungi and then assessed for bacterial or fungal growth. Qualitative assessment of antibacterial and antifungal activity was carried out at 24 h and 1-week post-treatment.

3.21 Qualitative Determination of Antibacterial and Antifungal Activity of Emulsion Treated Polyester and Cotton Fabric post 40°C Wash

Treated polyester and cotton fabric samples and untreated (control) samples were washed on a standard 40°C wash using 4.4 g of sodium perborate tetrahydrate and 24.08 g of ECE non-phosphate reference detergent (A) sodium perborate tetrahydrate (SDC enterprises, Bradford UK). The temperature of the wash cycle was measured using an iButton Thermocron temperature logger and data was read using OneWire Viewer software. After washing fabric samples were left to dry overnight and then the antibacterial and antifungal activity post-wash was determined against *E. coli*, *S. aureus*, *S. epidermidis* and *T. rubrum* using the method described in Section 3.20.

3.22 Qualitative Determination of Antibacterial Activity of Emulsion Treated Polyester and Cotton Fabric (Plate Count Method)

The antibacterial activity of treated fabric (polyester and cotton) against *E. coli*, *S. aureus* and *S. epidermidis* was determined using the plate count method adapted from BS EN ISO 20743:2013. The absorption method was employed by cutting six fabric (treated) and six control (untreated) samples of mass 0.40 ± 0.05 g and placing them into separate vials. Aliquots of 0.1 ml of bacterial working culture (10^7-10^8 CFU/ml) were pipetted at several points on each fabric sample.

At zero hour, immediately after inoculation, 10 ml of neutraliser was transferred into three of the inoculated test vials and three of the control vials which were mixed by shaking with a vortex mixer for 1 min x 5 cycles. The other three test and control vials were incubated at 37°C for 18-24 h and neutralised after incubation with 10 ml of neutraliser and mixed again by shaking with an incubator. Both shake-out solutions (0 h and 48 h) were serially diluted and plated on BHI agar and these were incubated at 37°C for 18-24 h after which colonies were observed and enumerated. The percentage reduction (R) was calculated using Equation (7):

$$\% R = \frac{(A - B)}{A} \text{ x}100 \quad (7)$$

Where *A* is the number of microorganisms in the shake-out solution for control cotton or polyester fabric samples immediately after inoculation (zero CT).

B is the number of microorganisms in the shake-out solution from test (treated) cotton or polyester fabric samples after each contact period (zero, 24 h or 48 h) before neutralisation.

3.23 Qualitative Determination of Antifungal Activity of Emulsion Treated Polyester and Cotton Fabric (Plate Count Method)

The antifungal activity of the treated fabric (polyester and cotton) against the dermatophyte *T. rubrum* was determined using the plate count method

adapted from BS ISO 13629-2:2014. *T. rubrum* spores inocula were prepared using the method described in Section 3.2.1. The absorption method was employed, and six treated fabric samples and six control (untreated) samples were cut and weighted to a mass of 0.40 ± 0.05 g and placed into separate vials. Then 0.1 ml of spore inoculum (10^7) was pipetted at several points on each fabric sample, for zero-hour test, immediately after inoculation, 10 ml of neutraliser was transferred into three test and three control vials and the solution was mixed by shaking with a vortex mixer for 1 min for five cycles. The remaining control and test samples were incubated at 30°C for 48 h and after incubation 10 ml of neutraliser was transferred into the vials and the solutions were again mixed by shaking. Both shake-out solutions (zero hour and 48 h) were serially diluted and plated on SDA, incubated at 30°C for 48 h – 7days to observe the fungal colony growth. After incubation the colonies were enumerated. The percentage reduction was calculated using Equation (7).

3.24 Antibacterial and Antifungal Activity of a 1% CS EO-Emulsion (Time-Kill Assay)

The antibacterial and antifungal activity of the EO emulsion (1% CS) against *E. coli*, *S. aureus* and *S. epidermidis* was assessed using a time-kill assay. An aliquot of 0.1 ml of emulsion was mixed with 0.1 ml of DMSO to solubilise the emulsion. This solution was added to 9.7 mL of SDW (test solution), mixed and then the solution was inoculated with 0.1 mL of working

bacterial/fungal culture (10^7 – 10^8 CFU/mL). Aliquots (1 ml) of this (test mixture) were added immediately (zero CT) into 9 mL of neutraliser to inactivate the emulsion and the remaining test solution was incubated at 37° for bacteria or 30°C for fungi. The test mixture was neutralised again after a contact period of 5 min for bacteria and after 10 min, 20 min, 30 min, 40 min, 50 min, 60 min and 120 min for the dermatophyte *T. rubrum*. Neutralised test solutions were serially diluted, plated and incubated at 37°C for 18-24 h (bacteria) or 30°C for 7 days (fungi) for observation and enumeration. The time-kill assay was conducted as above without the presence of the antimicrobial emulsion as a control.

3.25 Evaluation of Repellency of Litsea-Lemon EO Blend and Emulsion Treated Cotton Fabric against *Aedes aegypti* Mosquitos

The repellency of 10x10 cm cotton samples impregnated with 400 µl litsea-lemon EO blend and cotton samples padded with a 30 % litsea-lemon EO was assessed against female mosquitos belonging to the species *Aedes aegypti* using the Olfactometer test. An acrylic Olfactometer unit (Ross Lifescience, India) was used, comprised of a clear cylinder (base leg), equipped with a holding port and connected to two test branches (chambers) each equipped with a trapping port; one chamber designated for the control and the other for the treatment test. Modifications from WHO original dimensions were made to the Olfactometer, these can be seen in Appendix IV. During the test, odours in the air diffuse into

the branches through the base leg and its holding port, from which the mosquitos are released upon starting the test (WHO, 2013).

The Olfactometer unit was assembled in a 30-meter cubic room chamber equipped with a central top exhaust fan for decontamination of the air within the chamber. Clean air lines supplied both the test and control ports of the Olfactometer with clean airflow; this airflow was checked with an anemometer prior to starting the test and was measured to be 0.20 ± 0.05 m/s; air velocity through the base leg of the cylinder was measured to be 0.40 ± 10 m/s. The Olfactometer test chambers were checked for contamination before each test and repeats by performing a test under chemical-free conditions; ten mosquitos were released and checked for knock-down for a duration of 10 minutes. After the exposure time, if no knock-down was recorded, the Olfactometer was considered ready for use.

The repellency test was carried out by releasing ten *A. aegypti* female mosquitos (12-hour starved) through the base leg of the Olfactometer using aspirators, the mosquitos were allowed to acclimatize for 15 minutes without treatment. After acclimatization, treated (1% CS litsea-lemon EO emulsion) and control (impregnated with 400 µl of litsea-lemon 1:2 EO blend) 10 x 10 cm cotton samples were placed in the test chamber and control chamber respectively and their trapping ports opened for 30 seconds to allow the mosquitos to migrate to either of the aforementioned chambers. After which, the holding port was closed and the number of mosquitos that migrated towards the control chamber or

treatment chamber was recorded every minute for a total of 3 minutes. Mosquitos that were physically injured and/or incapable of flying or walking were ignored in the results. Each test was repeated three times and the Olfactometer unit was cleaned with 10% isopropyl alcohol and clean airflow passed through for 3 minutes before each new test.

The percentage of mosquitos repelled to the treatment port was calculated by dividing the number of mosquitos in the trapped in the treatment (test) port by the overall number of mosquitos in the test, according to Equation (8):

$$Percentage\ (\%)\ Repellency\ = \frac{MC - MT}{MC + MT}\ x\ 100 \qquad (8)$$

Where, *MC* is the number of mosquitos in the control port and *MT* is the number of mosquitos in the treatment port (WHO, 2013). The method was conducted by Ross Lifescience Private Limited, Maharashtra, India.

Chapter 4. The Antimicrobial Activity of Ten EOs Against Skin Infection Related Pathogens

4.1 Introduction

4.1.1 *Skin Infections*

The skin, the largest organ in the human body, is an ecosystem whose primary role is to act as a physical barrier that protects the body from the external environment, preventing attack from foreign microorganisms and toxic substances. The stratum corneum (the uppermost layer of the skin) produces a hostile environment for microorganisms, by the secretion of acidic sebum and the constant shedding of keratinocytes that make up the stratum corneum, leading to the removal of microorganisms in the process (Grice and Segre, 2011; Wilson, 2005). The skin consists of microorganisms that are present on a semi-permanent basis without causing disease (the natural microflora); they also provide protection by competing with invading microorganisms for space (attachment sites) and available nutrients, producing metabolic compounds and lowering the pH of the environment which inhibits the growth of microorganisms. Those most commonly found microorganisms on the skin's surface belong to the genera *Corynebacterium* spp.*, Staphylococcus* spp.*, Cutibacterium* (formerly *Propionibacterium*) spp., *Acinetobacter* spp.*, and Brevibacterium* spp. (Wilson, 2005).

When a break in the skin occurs (such as with burns, wounds, and ulcers), the defence becomes compromised and the skin can become colonised with microorganisms that are not indigenous to the skin and can lead to mild or even serious infections of the surrounding tissues (Dryden, 2009). The common SSTIs caused by microorganisms include impetigo, cellulitis (*S. aureus*), folliculitis (*P. aeruginosa, S. aureus*), ringworm, tinea pedis (*Trichophyton rubrum*) and acne (*Cutibacterium acnes*) among others (Orchard and van Vuuren, 2017). Non-infectious skin diseases like atopic dermatitis (eczema) can also cause pathogenic infections by damaging the skin and making eczema patients more at risk of colonization and secondary infections by *S. aureus* or the herpes simplex virus (Boguniewicz and Leung, 2013; Orchard and van Vuuren, 2017). It has been found that *S. aureus* plays a role in exacerbating eczema by producing super-antigens (Barnes and Greive, 2013; Gong et al., 2006; Jinnestål et al., 2014; Kozman et al., 2010; Petry et al., 2014).

It has been reported that 10% of hospital patients in the UK develop HAIs 9% of which are related to surgical wound infections (Abboud et al., 2014; Breathnach, 2013; Jhass et al., 2017). When wound healing does not progress normally, SSI can become a chronic wound with the bacterial infections affecting the healing rate. SSIs and chronic wounds can double the time a patient spends in hospital thus increasing the cost of healthcare, due to additional costs related to re-operation, extra nursing care, drug treatment and other indirect costs like patient dissatisfaction and litigation (NICE, 2017).

Typically, skin infections are treated topically, however, the ability of microorganisms to become resistant to antimicrobials like antibiotics, due to overuse and misuse of drugs has led to the emergence of antibiotic resistant bacteria such as MRSA, and multi-drug resistant Gram-negatives like *Pseudomonas* spp*., Escherichia* spp. and *Klebsiella* spp. which have created problems in the management of wound infections, by reducing the number effective antibiotics. Therefore, treatment is often challenging and unsuccessful, requiring the development of new agents active against these resistant strains of bacteria (Abboud et al., 2014; Breathnach, 2013; Visavadia et al., 2008).

Fungal infections have not been prioritized within medical, clinical and laboratory research, but these infections are now occurring more frequently among immunocompromised cancer patients and transplant patients. Though considered superficial infections, they can have serious psycho-social effects and can no longer be viewed as a ‘cosmetic’ issue (Cassella et al., 2002). Dermatophyte diseases, such as athlete’s foot, are also affected by the development of drug resistance and there are concerns with toxicity of the commonly used antifungal drugs, like azole derivatives, which can have adverse effects to patients, including hepatoxicity and gastrointestinal disturbances and have long treatment durations (Regev-Shoshani et al., 2013). The issues related to skin infections, their prevention and treatment have sparked interest in natural products within the UK and around the world, one of the oldest types of medicine, as an alternative to standard pharmaceutical products.

4.1.2 *EOs*

Plant products have often been used as alternatives to medicines over the years and EOs are one of the most popular natural products, often used in dermatology; EOs are often used in the treatment of fungal skin infections and generally their popularity has been growing both in the United Kingdom and in the United States (Bakkali et al., 2008; Nazzaro et al., 2013; Solórzano-Santos and Miranda-Novales, 2012). Orchard and van Vuuren (2017) identified that 62% of EOs are used for the treatment of infections caused by bacteria, fungi or viruses; 20% of EO use was attributed to skin conditions such as dermatitis and eczema while the remaining use (18%) was linked to general skin maintenance (Orchard and van Vuuren, 2017).

EOs are oily volatile and aromatic liquids that are formed in the organs of plants, commonly concentrated in specific regions such as leaves, fruit or bark. The antimicrobial properties of EOs have been known for centuries and their activity is directly correlated to the presence of bioactive compounds (alcohols, terpenes, esters, ketones, amines, sulphides and aldehydes), which are also responsible for the plant's characteristic odour and flavour (Calo et al., 2015; Patel and Gogna, 2015). Some of these molecules are naturally present in the plant, whilst others are activated by enzymes as a mechanism of self-defence in response to biotic or abiotic stress (Nazzaro et al., 2013). Lemon EO is part of the family of Rutaceae; it is obtained from *Citrus limon* peel by cold pressing and its components include limonene, citral and linalool (Ali et al., 2015; Misharina et

al., 2010; Peng et al., 2013). Citrus EOs are usually comprised by a mixture of components, including terpenes, aldehydes, alcohols and esters. The main component of citrus EOs is limonene, and other significant components include, α and β- pinene, γ-terpinene, terpinolene and sabine (Amorim et al., 2016). *Litsea* is an important genus in the *Lauraceae* family, found frequently in Asia, Austria and North and South America (Wong et al., 2014; Yang, K. et al., 2014). Litsea EO is extracted from *L. cubeba*, an evergreen tree found in Asia (Li et al., 2014; Zhang, 2012). It is used in traditional Chinese medicine for its carminative, diuretic, expectorant, stimulant, sedative and antiseptic properties; additionally, it has been found to have insect repellent activity (Yang et al., 2014). Its main component is citral (also referred to as neral or geranial); a study found that citral made up 87.4% of the overall litsea EO composition (Si et al., 2012). *R. officinalis* is an aromatic plant that belongs to the Lamiaceae family (Ali et al., 2015; de Barros Fernandes et al., 2014; Okoh et al., 2010; Ramos-e-Silva and Ribeiro de Castro, 2002).

EOs can be used in blends/combinations of two or more EOs with the goal of creating a synergistic antimicrobial or therapeutic effect which is greater than the sum of the individual oils. The synergistic antimicrobial effect observed in some EO blends can be achieved through the following mechanisms: (1) enhancement of solubility and bioavailability; (2) EO could affect multiple targets of the bacterial cell; (3) inhibition of the mutation mechanism of the bacterial cell to the EO; or (4) inhibition of bacterial cell efflux pumps, resulting in accumulation of the EO inside the cell (Hemaiswarya et al., 2008; Orchard et al., 2017).

Previous studies have found that EOs can create a synergistic antimicrobial effect when combined with other EOs and with other antimicrobials, and numerous studies have focused on blends of various EOs with mainly lavender, oregano or frankincense (de Rapper et al., 2012; Gutierrez et al., 2008; Orchard et al., 2017).

4.1.3 *EOs as Antimicrobials*

Many EOs have been found to have antibacterial and antifungal activity. A study by de Rapper et al (2013) found that lemon EO had activity against *S. aureus* at MIC of 3 mg/mL, though a different study by Prabuseenivasan et al (2006) reported MIC>12.80 mg/ml (de Rapper et al., 2013; Prabuseenivasan et al., 2006). Patchouli and geranium EOs have also been found antimicrobial against *S. aureus* with reported MICs of 1.7 µl and 5.4 respectively (Kwiatkowski et al., 2017). EOs have been able to also inhibit spore-forming bacteria like *C. difficile,* Mooyottu et al (2017) reported a significant reduction in sporulation (p<0.05) of *C. difficile* caused by carvacrol, a component found in some EOs like oregano and thyme (Mooyottu et al., 2017). Black seed oil has shown antimicrobial activity at 2% concentration against *B. cereus* and 2 strains of *C. difficile*, with ZOIs ≥ 15 mm (Aljarallah, 2016).

Citrus citratus (lemongrass) EO was found effective against fungi *Aspergillus niger*, *Aspergillus flavus* and *Penicillium verrucosum* with MICs

ranging from 0.006-0.03% (Viuda-Martos et al., 2008). Fennel seed EO has shown good antimicrobial activity against *T. rubrum, T. mentagrophytes, Trichophyton tonsurans* and *Microsporum gypseum* with MICs of 0.039 µl/ml and MFCs 0.078 µl/ml against both *T. rubrum* and *T. tonsurans* and MICs of 0.078 µl/ml and MFCs of 0.156 µl/ml against *T. mentagrophytes* and *M. gypseum* (Zeng et al., 2015).

Studies have evaluated the MICs of EOs against *P. aeruginosa* using the microdilution assay, reporting high MICs when using clove (5% v/v), lavender (>5% v/v), red thyme (>5% v/v), tea tree (5% v/v) EOs (Kavanaugh and Ribbeck, 2012). A study on the activity of *Allium sativum* (garlic), *Cinnamomum cassia* (Chinese cinnamon) and peppermint EOs against *P. aeruginosa*, reports MIC and MBC values of >0.5% v/v each for peppermint EO and MIC/MBCs of 0.5%/>0.5% v/v respectively for garlic EO (Lang et al., 2016). The activity of 17 EOs on *P. aeruginosa* demonstrated that 14 of those exhibited low antimicrobial efficacy against the organism, with MIC and MBC ranging between 1.8 – 3.6% v/v. However, good activity against Gram-negatives has been observed by Cinnamon EOs; Barbosa et al (2015) demonstrated that *Cinnamomum zeylanicum* inhibited both *E. coli* and *P. aeruginosa* at low MIC values of 0.25 mg/ml 0.80 mg/ml respectively (Barbosa et al., 2015). Cinnamon bark EO has also demonstrated inhibition of various isolated strains of *P. aeruginosa* with the lowest MIC observed of 0.056% v/v and the highest MIC 0.225% v/v (Utchariyakiat et al., 2016). This strong activity by cinnamon EO against *P. aeruginosa* has also been

reported on a study on various EOs from plants, with an MIC ≤1 µl/ml, though the antibacterial assay was only carried out in duplicate (Tarek et al., 2014).

4.1.4 *Methods for Determining the Antimicrobial Activity of EOs*

The first step in assessing the use of EOs for functional textile, is to determine whether they have any antimicrobial activity and to what degree; the diffusion method is one that is often employed in the screening of EOs. Two types of diffusion methods can be employed, the disc diffusion method is the most popular due to its simplicity, involving impregnating a filter paper disc with a known amount of EO which is then placed on the surface of previously inoculated agar. This is a popular method which has often been used for antibiotics, however, the volatile and hydrophobic nature of EOs could interfere with results and is therefore only used as a preliminary indication of activity. The agar diffusion method is the second type of diffusion method in which the paper disc is replaced by wells within the agar, into which the EO is inserted. The inhibition of the organism (or lack-thereof) is then observed after incubation of the plates, and the EO activity is determined by the zone of inhibition. These screening methods are not quantitative and serve to only give an indication of which EOs possess antimicrobial properties. These diffusion methods are often used as a screening tool and EOs which give interesting results are then carried forward and tested using dilution methods (Orchard and van Vuuren, 2017).

Dilution methods allow the quantitative measurement of an organism's susceptibility to a given EO; calculations of the MIC of the EOs being tested are then carried out, giving a better understanding of how potent an EO is against a given microorganism. The microdilution method assay uses a 96 well microtiter plate in which EOs are serially diluted using a solvent such as dimethyl sulfoxide (DMSO) or Tween 80 in the dilution process due to solubility issues arising from the hydrophobic nature of EOs. The results of the assay can be read visually or by reading the optical density (OD) and the MIC is chosen as the lowest concentration in which the growth of the microorganism being tested is inhibited. The macrodilution method replaces the 96 well plate with individual test tubes and though this method gives comparable results, it is time consuming, tedious and uses much larger volumes of test media. In the agar dilution method, the EOs are serially diluted using a solvent and each dilution is mixed with a known amount of molten agar, inoculated, poured into plates and incubated. The dilution plate that shows absence of growth post-incubation is regarded as the MIC. Various classifications of activity for EOs using dilution methods have been developed; Agarwal et al (2010), for example, regards concentrations of 1.00% or less to be noteworthy MIC values, which is what has been followed for this present investigation (Agarwal et al., 2010).

When bioactive drugs are combined, they have the potential to interact with a resulting synergistic, additive, indifferent or antagonistic effect. When a synergistic effect is observed, essentially a greater antimicrobial effect is observed when a combination of the substances is used compared to the

individual substances (Efferth and Koch, 2011). When a significant reduction in the antimicrobial effect of the substance is observed in combination compared to the individual substances, antagonism is conveyed (Hyldgaard et al., 2012). This effect, however, has been limited to the pathogen being studied, therefore, synergistic blends cannot be assumed to have the same effect on all organisms (Orchard and van Vuuren, 2017). To determine what interactions result from the combination of antimicrobials, methods such as the checkerboard method are used. In the checkerboard method, the antimicrobial substances are combined and serially diluted in microplates, with dilutions ranging above and below the MIC of the individual components (Langeveld et al., 2014). After incubation, the plate is assessed for combinations that are inhibitory, allowing determination of synergism or lack thereof between the combinations. To interpret data from the checkerboard method, the FIC index can be used in which the interaction is calculated according to Equation (1) when two-way interaction is assessed and Equation (2) when a three-way interaction is being assessed (Hossain et al., 2016; Orchard and van Vuuren, 2017; Stein et al., 2015). The FIC index method is one that is simple but with limitations, as it does not consider the different dose response two agents may have at the same concentration when 1:1 ratios are used. To evaluate this more accurately the isobole method can be used in which the contribution by each agent within a blend is evaluated along a line of additivity (a mathematical line); a straight line can be observed where there are indifferent or additive interactions, while a concave or convex curve will be observed for synergistic and antagonistic interactions respectively (Orchard and van Vuuren, 2017; Owen and Laird, 2018; Rather et al., 2013).

Unlike the checkerboard method, time kill assays allow time-dependent antimicrobial effects to be determined, as the number of surviving cells are enumerated at various time points; once a >2 $\log_{(10)}$ reduction is observed (compared to the most antimicrobial individual component) synergism is determined (Orchard and van Vuuren, 2017). The time-kill assay allows for greater reproducibility and sensitivity due to the quantitative nature of the results, but it is a time consuming and labour-intensive method (van-Vuuren and Viljoen, 2011).

In this chapter, ten EOs were screened for their MIC/MBCs and FICs against two Gram-negative bacterial organisms, *E. coli* and *P. aeruginosa* and two Gram-positive bacterial organisms *S. aureus S. epidermidis* and the dermatophyte *T. rubrum*.

4.2 Aims and Objectives

The overall aim of this chapter is to determine the antimicrobial efficacy of a range of EOs against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *T. rubrum* for potential use in a microencapsulation process.

Objectives:

- To screen a range of EOs against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *T. rubrum* using the disc diffusion method to determine their antimicrobial activity;
- To determine the MIC of the most antimicrobial EOs against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *T. rubrum,* using the microdilution method;
- To evaluate the synergistic relationship between the most antimicrobial EOs against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *T. rubrum* using FIC indices.

4.3 Results

4.3.1 *Screening EOs for Antimicrobial Activity*

The antimicrobial screening results show that *S. aureus* is the most sensitive bacterial organism to the EOs tested compared to *S. epidermidis*, *E. coli* and *P. aeruginosa,* with large zone of inhibition (>50 mm) observed for lemon, rosemary, rosewood and wild thyme (Table 4.1). The dermatophyte *T. rubrum* was the most sensitive organism overall, with eight out of the ten EOs tested resulting in complete inhibition (ZOI= 90.00 mm) of the fungi. Except bitter orange (ZOI= 81.17 mm) and sweet orange (ZOI= 46.50 mm). *P. aeruginosa* showed the greatest resistance to EOs; of the ten tested EOs, it was found to be resistant to rosewood, wild thyme, sweet orange and bitter orange EOs, with no zones of inhibition (ZOI) observed for each EO. Activity was observed by bergamot, citronella, lemon, litsea, peppermint and rosemary against *P. aeruginosa* but zones were significantly smaller compared to *S. aureus*, *E. coli*, *S. epidermidis* and *T. rubrum* ($p \leq 0.05$). Litsea, rosemary and lemon EOs exhibited the greatest antimicrobial activity against all the microorganisms tested, inhibiting *S. aureus* (ZOI 47.20 mm- 51.00 mm)*, P. aeruginosa* (ZOIs 20.20 mm-23.80 mm), *E. coli* (ZOIs 41.30 mm-90.00 mm) and *S. epidermidis* (ZOIs 27 mm– 90 mm) and the dermatophyte *T. rubrum* (ZOIs 90.00 mm), and were therefore carried forward to determine MIC, MBC/MFC and FICs. None of the EOs tested enhanced the growth of the organisms and example of EO zones can been seen in Figure 4.1.

Table 4.1 Mean ZOI (mm) of ten EO against *S. aureus, P. aeruginosa, E. coli, S. epidermidis* and *T. rubrum* using the disc diffusion method (n=6 + SD).

EO	*S. aureus**	*P. aeruginosa**	*E. coli**	*S. epidermidis**	*T. rubrum**
Bergamot	29.30 ± 2.20	20.00 ± 0.00	46.80 ± 10.10	47.33 ± 5.12	90.00 ± 0.00
Citronella	39.20 ± 2.20	20.30 ± 0.60	0.00 ± 0.00	90.00 ± 0.00	90.00 ± 0.00
Lemon	51.00 ± 7.60	21.30 ± 1.50	41.30 ± 4.00	27.00 ± 1.70	90.00 ± 0.00
Litsea	47.20 ± 3.70	20.20 ± 0.40	52.80 ± 2.90	90.00 ± 0.00	90.00 ± 0.00
Bitter Orange	25.00 ± 2.00	0.00 ± 0.00	28.70 ± 1.63	46.19 ± 1.36	81.17 ± 10.01
Sweet Orange	22.20 ± 0.80	0.00 ± 0.00	20.00 ± 0.00	25.35 ± 1.92	46.50 ± 3.73
Peppermint	47.80 ± 1.80	20.40 ± 0.50	0.00 ± 0.00	44.33 ± 8.02	90.00 ± 0.00
Rosemary	50.50 ± 12.90	23.80 ± 1.90	90.00 ± 0.00	29.50 ± 2.70	90.00 ± 0.00
Rosewood	50.3 ± 10.90	0.00 ± 0.00	90.00 ± 0.00	58.00 ± 4.69	90.00 ± 0.00
Wild Thyme	50.5 ± 12.80	0.00 ± 0.00	56.50 ± 6.20	43.33 ± 2.58	90.00 ± 0.00

*Controls were carried out for each organism to ensure growth was uninhibited by plain filter paper disk

Figure 4.1 Example of lemon EO disk diffusion zone against *S. aureus*

4.3.2 *Determination of MIC and MBC of EOs*

The lowest MICs and MBCs against all organisms tested were seen by litsea EO (Table 4.2) with the lowest MIC observed against *S. epidermidis* (MIC=0.6 µl/ml and MBC= 1.25 µl/ml). *P. aeruginosa* was still found to be the most resistant organism, with generally higher MICs and MBCs for the EOs compared to other organisms; litsea EO for example, was found to have an MIC of 10.0 µl/ml and an MBC of 20.0 µl/ml against the Gram-negative, which is noticeably higher compared to its MICs for the other organisms, such as *S. aureus* (MIC= 1.25 µl/ml and MBC= 2.5 µl/ml). Litsea was also the most effective of the three oils against the dermatophyte *T. rubrum;* its MIC against the fungus was 2.5 µl/ml and an MFC of 5 µl/ml, compared to lemon and rosemary which both had a higher MICs of 10 µl/ml and MFCs of 20 µl/ml. Overall, lemon EO was found to be the least antimicrobial against all four organisms compared to litsea

and rosemary EOs; against both *P. aeruginosa* and *E. coli* lemon EO was not bactericidal below 40 µl/ml. Against *S. aureus* and *T. rubrum* (the most EO-susceptible microorganisms during screening) MICs of 20 µl/ml and 10 µl/ml and MBCs of 40 µl/ml and 20 µl/ml respectively were observed by lemon EO (Table 4.2).

Table 4.2 MIC and MBC/MFC (µl /ml) of lemon, litsea and rosemary EOs against *S. aureus, P. aeruginosa, E. coli, S. epidermidis* and *T. rubrum* (n=6)

EO	***S. aureus***		***P. aeruginosa***		***E. coli***		***S. epidermidis***		***T. rubrum***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
Lemon	20	40	>40	>40	40	>40	20	40	10	20
Litsea	1.25	2.5	10	20	2.5	5	0.6	1.25	2.5	5
Rosemary	5	10	20	40	2.5	5	>40	>40	10	20

4.3.3 *Assessment of the Synergism between EOs*

Synergistic interactions could be observed when double combinations of the EOs were used (Table 4.3); all double combinations between lemon, litsea and rosemary EOs were synergistic against *S. aureus,* with a combination between litsea (0.6 µl/ml) and rosemary (0.10 µl/ml) EOs giving a synergistic FICI of 0.5. The combination of litsea EO (0.10µl/ml) with lemon EO (0.10 µl/ml) and the combination of rosemary EO (0.10 µl/ml) with lemon EO (10 µl/ml) were also found to be synergistic against *S. aureus*, with FICI of 0.09 and 0.5 respectively

(Table 4.3). Similarly, synergistic interactions were found between all double combinations of rosemary, lemon and litsea EOs against *E. coli*. A triple combination of rosemary (0.6 µl/ml), litsea (0.6 µl/ml) and lemon (2.5 µl/ml) was also found to have a synergistic effect against *E. coli* (Table 4.4). For *P. aeruginosa*, synergistic interactions were only found between litsea (2.5 µl/ml) and lemon (2.5 µl/ml) EOs (FICI 0.31); all other combinations were found to be either antagonistic or additive. No synergistic interactions were observed between double combinations of the EOs against *S. epidermidis* (Table 4.3) and between triple combinations of the EOs against *S. epidermidis*, *P. aeruginosa* and *S. aureus* (Table 4.4). Synergistic interaction between litsea (0.6 µl/ml) and lemon (2.5 µl/ml) oils was observed against the dermatophyte *T. rubrum* (Table 4.3). Noticeably, when synergism was observed, the MICs of the EOs combined had decreased, compared to the MIC of the EOs when they were used alone; MIC for litsea and lemon EOs against *S. aureus* for example were 1.25 and 20.0 µl/ml respectively (Table 4.2) but synergistic blend was found at a lower concentration of 0.10 µl/ml for both EOs respectively (Table 4.3). Blends of litsea and rosemary were also synergistic against *S. aureus* with a decrease in individual MICs from 1.25 µl/ml and 5.0 µl/ml respectively (Table 4.2) to MICs in combination of 0.60 µl/ml and 0.10 µl/ml for litsea and rosemary EOs respectively (Table 4.3).

Table 4.3 Antimicrobial interactions between double combinations of lemon, litsea and rosemary EOs against *S. aureus*, *P. aeruginosa*, *E. coli*, *S. epidermidis* and *T. rubrum* (n=6)

Organism	EO	MIC in combination (µl/ml)	FIC_{index}	Interaction
S. aureus	Litsea	0.60	0.50	Synergistic
	Rosemary	0.11		
	Litsea	0.10	0.09	Synergistic
	Lemon	0.10		
	Rosemary	0.10	0.50	Synergistic
	Lemon	10.0		
P. aeruginosa	Litsea	40.0	4.06	Antagonistic
	Rosemary	1.25		
	Litsea	2.5	0.31	Synergistic
	Lemon	2.5		
	Rosemary	10.0	0.75	Additive
	Lemon	10.0		
E. coli	Litsea	0.10	0.28	Synergistic
	Rosemary	0.60		
	Litsea	0.10	0.17	Synergistic
	Lemon	5.0		
	Rosemary	0.10	0.17	Synergistic
	Lemon	5.0		
S. epidermidis	Litsea	0.60	1.25	No interaction
	Rosemary	10		
	Litsea	1.25	2.15	No Interaction
	Lemon	1.25		
	Rosemary	1.25	0.75	Additive
	Lemon	1.25		
T. rubrum	Litsea	0.60	1.24	No Interaction
	Rosemary	10.0		
	Litsea	0.60	0.49	Synergistic
	Lemon	2.50		
	Rosemary	0.60	0.56	Additive
	Lemon	0.5		

Triple combinations of litsea, lemon and rosemary EOs only showed synergism against *E. coli* (FICI= 0.50); the three EO blend had an additive effect against *S. aureus* (FICI= 0.66) and *S. epidermidis* (FICI= 0.79), and a non-interactive effect against *P. aeruginosa* (Table 4.4). The isobologram for *E. coli* in Figure 4.2b shows a concave curve representing synergism when litsea (MIC in combination =0.60 µl/ml), lemon (MIC in combination=2.50 µl/ml) and rosemary (MIC in combination 0.60 µl/ml) blend was assessed. Isobolograms for *S. aureus*, *S. epidermidis* and *P. aeruginosa* show some concave curves at the end of two axis, representing the MIC of the individual oil; synergism between the three EOs is not observed, however, antagonism is clearly represented by the convex curves for *S. aureus*, *S. epidermidis* and *P. aeruginosa* (Figure 4.2).

Non-synergistic 3-way combinations of the litsea, lemon and rosemary EO blend were still antimicrobial against *S. aureus*, *P. aeruginosa*, *E. coli*, *S. epidermidis* and *T. rubrum* and no antagonism was recorded (Table 4.4), however, blends of litsea and lemon EO showed synergism against four out of five organisms tested, and did not show antagonism against *S. epidermidis* (Table 4.3).

Table 4.4 Antimicrobial interactions between triple combinations of lemon, rosemary and litsea EOs against *S. aureus*, *P. aeruginosa*, *E. coli* and *S. epidermidis* (n=6).

Organism	EO	MIC individually (µl/ml)	MIC in combination (µl/ml)	FIC_{index}	Interaction
S. aureus	Litsea	1.25	0.60		
	Rosemary	5.0	0.60	0.66	Additive
	Lemon	20.0	1.25		
P. aeruginosa	Litsea	10.0	10.0		
	Rosemary	20.0	20.0	2.03	Non interactive
	Lemon	>40.0	1.25		
E. coli	Litsea	2.5	0.60		
	Rosemary	2.5	0.60	0.50	Synergistic
	Lemon	40.0	2.50		
S. epidermidis	Litsea	0.6	0.10		
	Rosemary	>40.0	10.0	0.79	Additive
	Lemon	20.0	5.0		

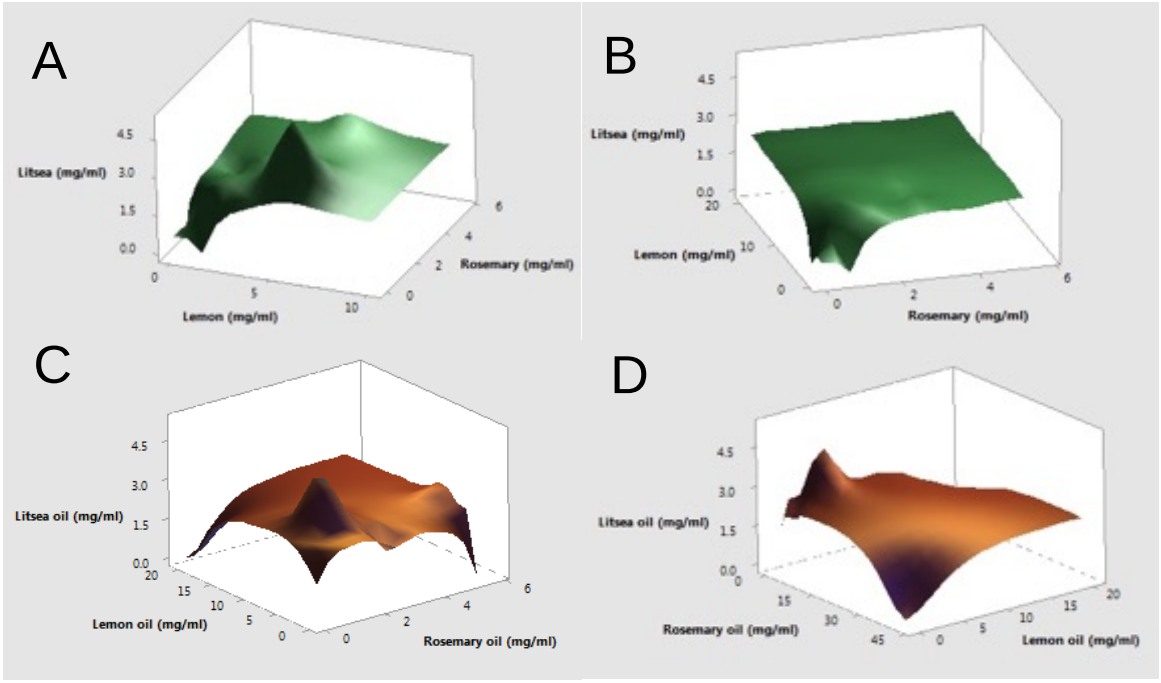


Figure 4.2 Isobolograms showing 3-way interactions of litsea, lemon and rosemary EOs against a) *P. aeruginosa*, b) *E. coli*, c) *S. aureus* and d) *S. epidermidis*

4.4 Discussion

Interest in investigating the antimicrobial ability of EOs has increased and their many potential uses have been explored in food preservation, pharmaceutics, natural therapies and cosmetics. With the aim of developing a highly active EO-based nanoemulsion, the antimicrobial and antifungal properties of ten EOs were compared against two Gram-positive bacterial species, (*S. aureus* and *S. epidermidis*), two Gram-negatives (*E. coli* and *P. aeruginosa*) and a dermatophyte (*T. rubrum*) associated with skin conditions. The potential of EOs for use in various applications is suggested by their broad spectrum of antimicrobial activity, which is also evident in this investigation as EOs such as *L. cubeba* (litsea), *C. limon* (lemon) and *R. officinalis* (rosemary) showed activity against all organism tested (Table 4.1), with ZOIs ranging between 20.20-90.00 mm for litsea EO, 21.30-90.00 mm for lemon EO and 23.80-90.00 mm for rosemary EO. The MICs were therefore determined for the antimicrobial activity of the 2 citrus EOs of litsea and lemon and the EO the rosemary herb.

S. aureus is commonly found in wounds, and though many studies on EO activity focus on *S. aureus* there are still many oils that have not been tested against *S. aureus* and its resistant strain; Orchard and van Vuuren (2017) argue that only 55% of the EOs documented from the aromatherapeutic literature for use in dermatological infections have been tested against *S. aureus*. EOs of *Boswellia* spp. (frankincense), *Eucalyptus* spp. (eucalyptus), *Lavandula augustifolia* (lavender), tea tree, *Mentha piperita* (peppermint), basil, *R. officinalis* (rosemary) and *T. vulgaris* (thyme) are among the EOs most tested against *S.*

aureus (de Rapper et al., 2013; Jiang et al., 2011; Orchard et al., 2017; Van Vuuren et al., 2010). A study on lavender EO and other aromatherapeutic oils reported noteworthy MIC values for various EOs against *S. aureus*, including vetiver (MIC= 0.75 mg/ml), bay laurel (MIC= 0.83 mg/ml) and Indian sandalwood (MIC= 0.25 mg/ml) EOs (de Rapper et al 2013). *L. cubeba* was also tested in this study and was able to inhibit *S. aureus* growth at an MIC of 1.50 mg/ml, which notably was the same MIC against *P. aeruginosa* (de Rapper et al., 2013). In the present study, litsea EO exhibited higher antimicrobial activity than rosemary and lemon EOs; moderate activity was observed by litsea EO (Table 4.2) against *E. coli* and *T. rubrum*, with MIC and MBC/MFCs of 2.5 µl/ml and 5 µl/ml respectively for both organisms, however, stronger activity was observed against *S. epidermidis* (MIC= 0.6 µl/ml) and *S. aureus* (MIC= 1.25 µl/ml). Comparable results were generated in a study on leaf and fruit EOs of *L. cubeba*, in which an MIC of 1.25 mg/ml was observed against *S. aureus*, while an MIC of 10 mg/ml was recorded against *E. coli* (Saikia et al., 2013). Knowledge on the antimicrobial activity of *L. cubeba* (litsea) EO against microorganisms like dermatophytes and bacteria is still limited; a study on its activity using the toxic food method, showed that litsea EO was able to prolong the lag phase of *E. coli* for approximately 12 hours at a concentration 0.0625% v/v, while at a concentration of 0.125% v/v, *E. coli* cells were killed within 2 hours, as shown by TEM (Li et al., 2014). *S. epidermidis* has been somewhat neglected in EO studies, possibly due to the lack of perceived threat, however, MRSE has become more problematic recently due to antimicrobial resistance (Opalchenova and Obreshkova, 2003). Numerous studies have assessed the activity of tea tree oil against *S. epidermidis*, and a

study by Yoon et al (2009) found that the EO was able to inhibit two antibiotic resistant strains of *S. epidermidis* (SK9 and SK19) at an MIC of 1.00 µl/ml against both strains (Bajpai et al., 2009).

Dermatophytosis is a contagious fungal infection caused by a group of fungi known as dermatophytes; these filamentous fungi infect areas of the body that are rich in keratin and the dermatophyte *T. rubrum* has been previously been identified in studies as the major causative agent (n=39, 49.37%) isolated in dermatophyte related infections (Babayani et al., 2018). Symptoms of a dermatophyte infections are not usually life threatening, but treatment can be challenging, costly and cause side effects. Many anti-fungal agents are available, including azoles such as ketoconazole, fluconazole and itroconazole, but can present side effects such as those seen with the use of ketozonazole, which include nausea, abdominal pain and itching; when used long-term such drugs can be toxic thus limiting their use, this and the high cost of treatment signify a need to focus on development of new suitable alternatives to these antifungal agents (Ouf et al., 2016; Shin and Lim, 2004). Tinea pedis is usually treated topically therefore EO are suitable candidates for treatment but very few research and clinical studies have been dedicated to dermatophytes like *T. rubrum*, their infections and their susceptibility to EOs. Shin and Lim (2004) assessed the inhibition of *T. rubrum* by herb EOs by the disc diffusion method and found rosemary and thyme EO had inhibition zones of 33.0 ± 1.15 mm and >39.0 mm respectively; MIC /MFC observed for thyme was 1 mg/ml, while that of rosemary was 8 mg/ml (Shin and Lim, 2004). In contrast, thyme and rosemary EO in this

present study (Table 4.1) gave total inhibition of *T. rubrum* (90 ± 0 mm); MIC and MFC of both rosemary and lemon EOs against *T. rubrum* were 10 µl/ml and 20 µl/ml respectively (Table 4.2). Comparable results have been reported for rosemary EO against *T. rubrum* with MIC and MFC of 8 mg/ml respectively (Shin and Lim, 2004). Sharma and Tripathi (2008) explain that the mode of action of EOs against dermatophytes involves loss of rigidity and integrity of the cell wall which causes loss of the cytoplasm and its components, eventually resulting in death of the mycelium (Sharma and Tripathi, 2008). Much better activity was seen with litsea EO in this study, exhibiting up to a 4 times lower MIC and MFC of 2.5 µl/ml and 5.0 µl/ml respectively compared to lemon and rosemary EO. Similarly, a south African study on 59 commercial EOs reported *L. cubeba* (litsea) EO to have the best anti-dermatophyte activity, with a notable MIC of 0.09 mg/ml against *T. mentagrophytes*, compared to lemon (MIC=0.38 mg/ml) and rosemary (MIC = 0.75 mg/ml) EOs (Orchard et al., 2017). Fennel EO has also demonstrated ability to inhibit dermatophytes growth; its activity was assessed against 3 strains of *T. rubrum* with MIC and MFC and 0.039 µl/ml and 0.078 µl/ml reported against all 3 strains tested (Zeng et al., 2015). The difficulty in treating dermatophyte infections should be an incentive in further investigating EOs that show strong anti-dermatophyte activity; a randomized, controlled, double-blinded study on the treatment of interdigital tinea pedis using tea tree EO reported a mycological cure rate of 64% for those using a 50% tea tree EO solution twice daily for a four-week period, compared to a rate of 31% for the placebo group (Satchell et al., 2002). It would therefore be useful to observe more clinical studies on EOs such as litsea EO which prove to have strong antifungal activity.

The most robust of the organisms tested was *P. aeruginosa,* with no antimicrobial activity observed (Table 4.1) for sweet orange, bitter orange and wild thyme EOs. This is consistent with previous studies which have reported no inhibitory activity of orange EO and thyme EO (no inhibition zone diameter) against *P. aeruginosa* (Mahboubi et al., 2018; Vieira-Brock et al., 2017). Screening results in this investigation (Table 4.1) showed susceptibility of *E. coli* to herb EOs such as wild thyme and rosemary (56.5 mm and 90 mm respectively). This activity was also seen in findings by Sienkiewicz et al (2016) with thyme and rosemary EOs giving smaller ZOIs of 23 mm and 12 mm respectively, though the quantity of EOs used to impregnate the disks and the size of disks used is not mentioned in the study (Sienkiewicz et al., 2017). The antimicrobial activity of rosemary EO has also been assessed against a range of *P. aeruginosa* isolates with reported MIC values ranging between 5 and 40 µl/ml (Araby and El-Tablawy, 2016; Gomes et al., 2012).

Generally higher MICs were observed in the present study against *P. aeruginosa* for lemon, litsea and rosemary EOs (>40 µl/ml, 10 µl/ml and 20 µl/ml respectively) compared to other organisms (Table 4.2). Gram negative pathogens like *P. aeruginosa* and *E. coli* are of critical concern in the field of drug resistance being able to colonise wounds and cause infection (Alonso-Isa et al., 2017; Cefalu et al., 2017). The low sensitivity of *P. aeruginosa* to EOs has also been observed in studies on various EOs against *P. aeruginosa*, with tested EOs of lemongrass, peppermint, caraway, anise, fennel, geranium, clove, and

lavender EOs having MICs >16 µl/ml the maximum concentration tested (Tarek et al., 2014).

EOs are said to be able to penetrate the cell wall and cytoplasmic membranes of bacteria by disrupting the integrity of various layers of the membranes, which leads to disruption of the cell (Saikia et al., 2013). The generally low sensitivity to most EOs exhibited by Gram-negatives like *P. aeruginosa* has often been attributed to the lipopolysaccharide (LPS) rich outer membrane which serves to protect the cytoplasmic membrane against antimicrobial activity, increasing tolerance to hydrophobic EOs compared to Gram-positive organisms (Calo et al., 2015; Nazzaro et al., 2013; Zhang et al., 2016). This is not always the case, as in this present study, the other Gram-negative organism tested, *E. coli,* did not experience the same degree of resistance as *P. aeruginosa* to the same EOs. In the case of litsea EO, for example, an MIC of 2.5 µl/ml was required to inhibit *E. coli,* while *P. aeruginosa* showed greater resistance (MIC 10 µl/ml) by the same EO (Table 4.2). A study by Orchard and van Vuuren (2017) on commercial EOs reported equal sensitivity for Gram-positive *S. aureus* and Gram-negative *E. coli* to *L. cubeba* (litsea) EO (MIC= 1.00 mg/ml), whilst *P. aeruginosa* was more resistant (MIC= 1.50 mg/ml). Additionally, the study found Gram-negative *E. coli* and *P. aeruginosa* to be equally susceptible as Gram-positive *S. epidermidis* and *S. aureus* strains to *Eucalyptus globulus* (eucalyptus) EO (MIC= 2.00 mg/ml), *Cinnamomum zeylanicum* (cinnamon) EO (MIC= 1.00 mg/ml), *Laurus nobilis* (bay) EO (MIC=

1.00 mg/ml) and *R. officinalis* (rosemary) EO (MIC=2.00 mg/ml; Orchard et al., 2017).

In this present study, the focus was on assessing the potential activity of EOs used on their own and within blends rather than in conjunction with traditional antimicrobials as EOs can have the advantage of creating stronger antimicrobial activity of individual oils when used in a blend; litsea and lemon EO blend, in this present study, showed synergistic effect against *P. aeruginosa* (FICI= 0.31) at a 1:1 ratio of 2.5 µl/ml concentration for both EOs. When this is compared to individual MIC for the EOs used individually (>40 µl/ml for lemon and 10 µl/ml for litsea EO), it is seen that blends have the potential to decrease possible toxicity by lowering the required dose of the antimicrobial agent and demonstrates that synergy is not necessarily shown only by EOs which have strong efficacy independently (Orchard et al., 2017). Greatest success with the blends of the 3 tested EOs was seen against *S. aureus* with synergistic blends observed between double combinations for all 3 EOs (Table 4.3), with the most efficacious combination observed again between litsea and lemon at a 1:1 ratio (FICI= 0.09). A study investigated various 1:1 combinations of *L. angustifolia* (lavender) with other EOs and found that blends of lavender EO with *C. zeylanicum* (FICI= 0.50), *C. limon* (FICI=0.38), *Daucus carota* (FICI=0.50), *Juniperus virginiana* (FICI= 0.50) and *T. vulgaris* (FICI= 0.40) all gave synergistic results (de Rapper et al., 2013). In contrast, none of combinations of lavender and the 45 other EOs studied showed a synergistic effect against *P. aeruginosa*, while 31 out of the 45 combinations were synergistic against the fungus *C. albicans* (de Rapper et al.,

2013). It is therefore wrong to assume that synergistic EO blends seen against one pathogen will have the same effect on all; Orchard et al (2017) argues that synergistic antimicrobial activity is limited by the pathogen being tested (Orchard and van Vuuren, 2017). In this present study, for example, synergy was observed for litsea and lemon blends against dermatophyte *T. rubrum* (FICI= 0.49), Gram-negative *P. aeruginosa* (FICI= 0.31) and *E. coli* (FICI= 0.17) and Gram-positive *S. aureus* (FICI= 0.09), but all 2-way blends of litsea, lemon and rosemary resulted in additive and indifferent interactions against *S. epidermidis* not further enhancing the activity of the individual EOs (Table 4.3). Triple combinations of the EOs in this study only showed synergism against *E.coli* with only one concentration combination give this effect as can be observed in the isobologram (Figure 4.2b); the three EO blend did not appear to have any added benefit for *S. aureus*, *S. epidermidis* and *P. aeruginosa* and antagonistic interactions can be observed in the isobolograms for each organism (Figure 4.2). Blends of litsea and lemon EO showed synergism against four out of five organisms tested (Table 4.3) and were still antimicrobial against *S. epidermidis* though non-synergistic (FICI= 2.15). Thus the 3-way blends between litsea, lemon and rosemary EO were not carried forward for investigation. The MICs of litsea and lemon EO within the blend that were antimicrobial against each organism were 2.5 µl/ml and 5.0 µl/ml respectively, representing a litsea: lemon ratio of 1:2. This ratio was therefore carried forward into analysis and product development in Chapter 5 and Chapter 6.

In the present study, the greatest antimicrobial synergistic effect (Table 4.3) was observed between blends of litsea and lemon EO inhibiting the growth of dermatophyte *T. rubrum* (FICI= 0.49) at minimum concentrations of 0.60 µl/ml and 2.50 µl/ml for litsea and lemon oil respectively, within the blend. Few studies have focused on EO blends against dermatophyte pathogens; Cassella et al (2002) observed synergy between blends of *Lavandula agustifolia* (lavender) and tea tree EO against dermatophytes *T. mentagrophytes* (70% lavender/ 10% tea tree) and *T. rubrum* (10% v/v lavender/ 20% tea tree), resulting in complete inhibition (Cassella et al., 2002).

Gutierrez et al (2008) suggest that the reason why EOs with moderate to poor individual antimicrobial efficacy, can result in an enhanced effect when combined, such as seen with *P. aeruginosa* in this study, lie within the mechanism of action and the composition of the EOs, as combinations with other EOs containing different chemical compounds which could increase their activity (Gutierrez et al., 2008). The differences in activity of EOs in this study and those found in literature can be attributed to the chemical diversity of EOs and are therefore to be expected (Nguyen et al., 2016). Variation in chemical composition of extracts and EOs from the same plants, is due to the different climates and environmental conditions that the plants are grown in such as soil, acidity and harvest times. This means that EOs that have been harvested form different plants may show variation in antimicrobial activity; additionally, different strains of an organism may show a difference in susceptibility (Tarek et al., 2014). Thus, the major chemical components of *L. cubeba* and *C. limon* have been analysed

in Chapter 5. In this present study no anaerobic organisms were tested as they were not among the organisms of interest.

In conclusion EOs are undoubtedly promising natural antimicrobials which are able to inhibit the growth of Gram-positive and Gram-negative bacteria as well as dermatophytes such *T. rubrum*, for which there is a paucity of research in the published literature. The most effective EO (which worked against all four organisms) was litsea, with an average zone of inhibition (ZOI) of 4.7 cm against *S. aureus*, 2.0 cm against *P. aeruginosa*, 5.3 cm against *E. coli* and 4.3 cm against *S. epidermidis*. Lemon and rosemary EOs were the only other two EOs which were effective against *P. aeruginosa* with mean ZOI's of 2.1cm and 2.4 cm respectively. Litsea, lemon and rosemary EOs were therefore carried forward to determine minimum inhibitory concentrations (MICs) and fractional inhibitory concentrations (FICs); MICs observed by litsea EO were lowest for all organisms ranging between 1.25 – 10 µl/ml. Blends of litsea and lemon showed synergy against *S. aureus* (FIC index = 0.09), *P. aeruginosa* (FIC index= 0.31), *E. coli* (FIC index =0.17) and *T. rubrum* (FIC index = 0.49). Blends of citrus EOs lemon and litsea were found to be potential antimicrobial agents for inhibiting the growth of *S. aureus, P. aeruginosa, E. coli*, *S. epidermidis* and *T. rubrum* for the treatment of skin infections and showed that EO can inhibit bacteria individually and synergistically (thus lowering concentration and any possible toxicity), making them good candidates for use in functional antimicrobial textiles. The potential encapsulation of EOs for use in functional textiles such as wound

dressings that are eco-friendly could be used effectively towards the treatment and prevention of infectious skin conditions and malodour.

Chapter 5. Encapsulation of Antimicrobial EO-blend of Litsea and Lemon

5.1 Introduction

EOs such as those of litsea and lemon, are mixture of highly complex, natural and volatile compounds produced by aromatic plants as secondary metabolites, such as of citral (Figure 5.1) and limonene (Figure 5.2). Their complex make-up of individual aroma compounds protects the plants from microorganisms in their environment, insects and herbivores and these compounds provide EOs their bactericidal, fungicidal and viricidal properties (Orchard and van Vuuren, 2017). The rise in the prevalence of antibiotic resistant pathogens observed in recent years, has led to increased interest in the development of functional textiles with antimicrobial properties. Synthetic antimicrobial agents such as QACs, triclosan and metals have been used to develop textiles with durable and effective antimicrobial activity against a range of microorganisms, however, environmental concerns related to these synthetic compounds (such as water pollution, bioaccumulation and non-biodegradability) have become a cause for concern (Ali et al., 2014; Tawiah et al., 2016; Windler et al., 2013).

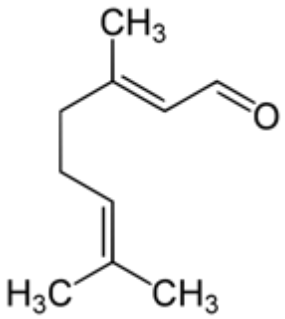


Figure 5.1 Chemical structure of citral compound

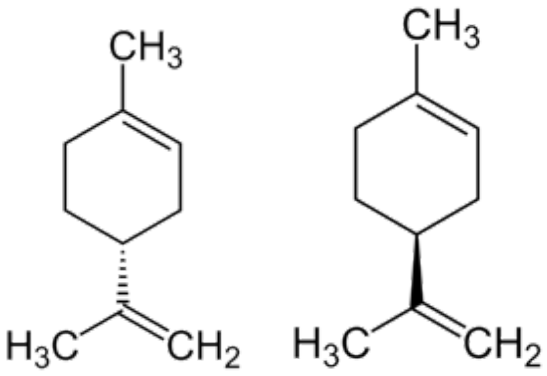


Figure 5.2 Chemical structure of Limonene isomers (R)-limonene (left) and (S)-limonene (right)

Natural, plant-based ingredients like EOs have grown in popularity as they represent an eco-friendly and biodegradable alternative for use in antimicrobial textile finishing. EOs have found various uses within the fields of textiles, foods, pharmaceuticals, cosmetics and agrochemicals, however, challenges are faced with the application of these natural antimicrobials due to their volatility, sensitivity to light and air (which can cause degradation) and hydrophobicity (Bakry et al., 2016). Suitable methods and formulations need to be employed with the aim of protecting the oil from volatilization and degradation, controlling its release rate and preventing unacceptable deterioration of the final product (Ali et al., 2014; Aziz et al., 2015; Bakry et al., 2016) For the successful formulation and

development of such EO-based products, microencapsulation can be a viable method that will preserve the functional and physicochemical properties of the oil and allow for greater durability of the final product (Aziz et al., 2015; Javid et al., 2014).

Microcapsules are based on a process by which small particles or droplets (the active agent) are coated by a natural or synthetic polymeric wall material or are embedded within a homogeneous or heterogeneous matrix (Bakry et al., 2016; Calvo et al., 2011; Rodrigues, Sofia et al., 2008). Various microencapsulation techniques have been developed, including emulsification, complex-coacervation, spray drying, *in situ* polymerization, interfacial polymerization, air suspension coating, centrifugal extrusion (Bakry et al., 2016; Calvo et al., 2011; Rodrigues et al., 2008). The microencapsulation technique to be employed will depend on the intended final application and the desired microcapsule characteristics, such as particle size and shape, biocompatibility, permeability and degradability (Aziz et al., 2015; Cheng et al., 2009). Techniques such as spray drying may be unsuitable for EOs, due to the high temperatures (up to 200°C) used in the drying chamber; additionally, Nazzaro et al. (2011) confirmed that oxidation of the oil can occur due to the increased oxygen availability during the atomization process (Bakry et al., 2016; Nazzaro et al., 2011). In this present study, the emulsification technique was used, as it allows for the encapsulation of the EOs in an aqueous final solution which can be used directly for easy and simple application on textiles. Emulsions are relatively simple to prepare, are low cost and do not involve the use of high temperatures.

Various EOs have been encapsulated via emulsification, including those of rosemary, thyme, oregano, clove, cinnamon and lime (Campelo et al., 2017; Hosseini et al., 2013; Mohammadi et al., 2015; Pecarski et al., 2014; Purwanti et al., 2018; Turasan et al., 2015)**.**

Many products ranging from pharmaceutical, food, textile and other industries are based on emulsions. Emulsions are mixtures of at least two immiscible liquids (phases), usually water and oil, in which one of the phases is dispersed as small droplets within the other. When the oil phase is dispersed within an aqueous phase it forms an oil-in-water (O/W) emulsion, however, when the water phase is dispersed in an oil phase, a water-in-oil (W/O) emulsion is formed. Complex emulsions can also be formed, such as water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsions (Aulton and Taylor, 2017; Bakry et al., 2016; Campelo et al., 2017).

Biopolymers, specifically natural occurring polysaccharides like chitosan and alginates, are becoming popular carriers in encapsulation processes. The deacetylated form of chitin, chitosan, has been used to protect compounds like EOs which are sensitive to temperature, oxygen and light, using methods like ionic gelation (Xu and Du, 2003) and spontaneous emulsification (Wilson et al., 2010). Carvacrol-loaded chitosan nanoparticles were prepared using a two-step method combining emulsification and ionic gelation, and the resulting particles effectively inhibited the growth of *E. coli, S. aureus* and *Bacillus cereus* with an MIC of 0.257 mg/mL and MBC of 8.225, 4.113 and 2.056 mg/ml respectively

(Keawchaoon and Yoksan, 2011). Chitosan has also been used to successfully encapsulate oregano EO, showing a two-phase release profile (Hosseini et al., 2013). A study on the microencapsulation of citronella EO discovered that increasing the concentration of chitosan in the encapsulation significantly affected the encapsulation efficiency of the EO by decreasing it and that homogenization speed affected particle size (Hsieh et al., 2006).

Alginates are natural polymers extracted from brown algae and have been widely used in the form of sodium alginate to encapsulate pharmaceutical actives and EO's such as clove, thyme and cinnamon (Shinde and Nagarsenker, 2011; Soliman et al., 2013). The formulation of sodium alginate particles is achieved through cross-linking, such as ionic cross-linking; a study on lemon balm-loaded sodium alginate beads cross-linked with calcium chloride found that there was no interaction with the extract and its antioxidant activity was not affected by the encapsulation (Najafi-Soulari et al., 2016). Interest in polymers such as chitosan and sodium alginate have received interest in food, pharmaceutical and biomedical applications, such as drug delivery systems due to their favourable biodegradable, biocompatible and mucoadhesive properties (Pedro et al., 2009).

Due to the rise in antibiotic resistance, the ecological concern created by current synthetic antimicrobials and the increased demand for eco-friendly antimicrobials and textile products, the development of 'green' formulations based on natural antimicrobials such as EOs and natural formulation ingredients such as biopolymers must explored, with the aim of developing safer, yet capable

functional textiles. The antimicrobial blend of litsea and lemon EOs is a potentially ideal alternative to synthetic compounds and the combination of biopolymers such as sodium alginate and chitosan are good candidates for a natural formulation that can be subsequently applied on textiles.

5.2 Aims and Objectives

The aim of this chapter was to develop a formulation that would encapsulate an EO blend of *C. limon* (lemon) and *L. cubeba* (litsea) using an eco-friendly technique suitable to use in coating textiles to render them antimicrobial.

Objectives:

- To encapsulate EOs within formulations that are eco-friendly and stable;
- To assess the stability of the formulations both qualitatively and quantitatively;
- To characterise and determine the physicochemical characteristics of the emulsions and its components including particle size, viscosity and thermal behaviour;
- To determine the release kinetics of EOs from the formulation.

5.3 Results

5.3.1 *Antimicrobial Activity of Litsea-Lemon EO O/W Emulsions*

The *in vitro* antimicrobial activity of the final o/w formulations was assessed against *E. coli*, *S. aureus* and *S. epidermidis* (Table 5.2); it was observed that emulsions with a final concentration (FC) of chitosan greater than 1.0 % w/v were not able to inhibit the growth of *S. aureus* and *S. epidermidis*. The growth of *E. coli* was inhibited, although the ZOI decreased with increasing amounts of chitosan with a ZOI of 31.36 ± 4.12 mm for a chitosan FC of 1.25% w/v reducing to a ZOI of 16.41 ± 14.21 mm at the highest chitosan FC of 2.0% w/v. Formulations with CS FC ranging from 0.125 – 1.0% w/v inhibited all bacterial strains with ZOIs ranging from 22.73 mm to 46.09 mm (Table 5.2)

Table 5.1 Antimicrobial activity (inhibition zone, mm) of 30% litsea-lemon EO O/W emulsions of varying chitosan concentrations against *S. epidermidis*, *E. coli* and *S. aureus*. Disk diffusion (-) not carried out.

Batch No.	**Chitosan (%w/v)**	***S. epidermidis* (mm)**	***E. coli* (mm)**	***S. aureus* (mm)**
F1	2.00	0.00 ± 0.00	16.41 ± 14.21	0.00 ± 0.00
F2	1.25	0.00 ± 0.00	31.36 ± 4.12	0.00 ± 0.00
B1	1.00	25.37 ± 1.64	38.00 ± 2.23	22.73 ± 1.15
B2	0.75	24.35 ± 0.40	39.00 ± 1.50	24.82 ± 1.01
B3	0.50	28.17 ± 2.75	44.00 ± 0.35	25.65 ± 0.67
F6	0.25	29.87 ± 3.86	46.09 ± 1.55	28.41 ± 1.15
F7	0.125	28.29 ± 4.20	41.83 ± 4.60	28.30 ± 2.50
F8	0.05	-	-	-

The antimicrobial activity of the individual components outside of the emulsion were also assessed against *E. coli*, *S. aureus* and *S. epidermidis* (Figure 5.3). Results show that a 1% w/v chitosan solution possessed antimicrobial activity against all bacterial strains tested with mean ZOIs of 7.54 mm, 7.99 mm and 8.46 mm against *E. coli*, *S. aureus* and *S. epidermidis* respectively. When comparing the EO blend ZOI with those of standards of the major compounds, citral showed greater ZOIs, ranging from 25.00-30.05 mm*;* in comparison both S- and R-limonene had lower ZOI, with their largest zones being 10.63 mm and 18.54 mm respectively. The EO blend showed similar activity to that of pure citral standard with mean ZOIs ranging from 24.04 – 34.94 mm; no significant difference was observed between ZOI by the EO blend and those by citral ($p \geq 0.05$). Results from the disk diffusion (Figure 5.3) also showed that a 1% w/v solution of sodium alginate did not possess any antimicrobial activity against *E. coli*, *S. aureus* and *S. epidermidis* whilst there is an apparent small contribution of antimicrobial activity by chitosan, giving mean ZOIs of 7.54 mm, 7.99 mm and 8.47 mm respectively.

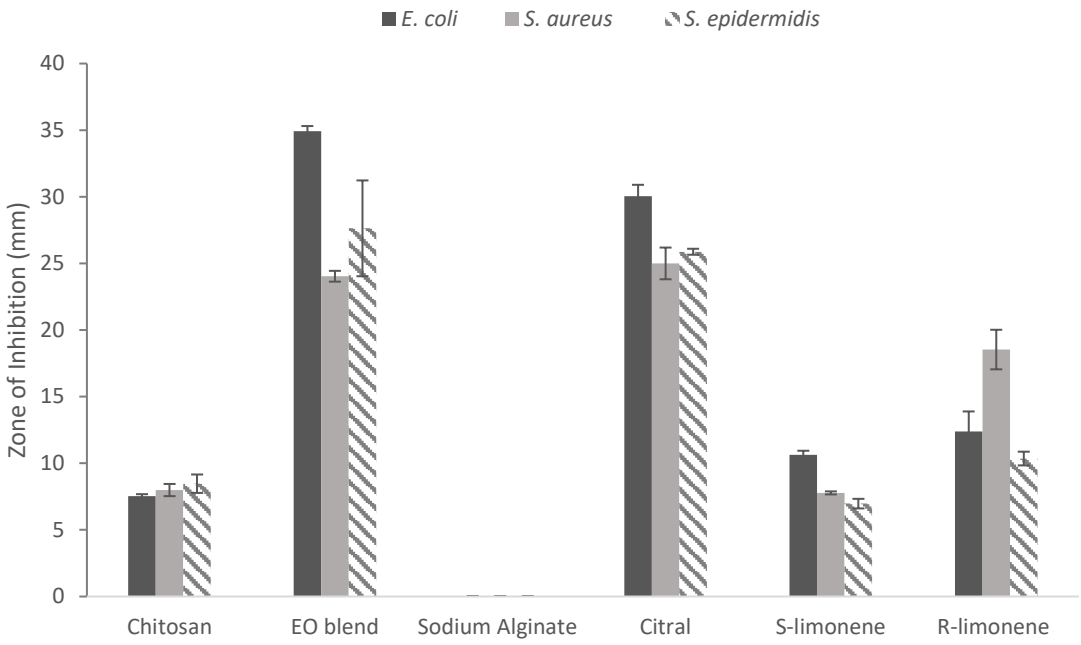


Figure 5.3 Mean Zones of inhibition (mm) of chitosan (1% w/v), litsea and lemon EO blend (1:2), sodium alginate (1% w/v) citral, S-limonene and R-limonene against *E. coli*, *S. aureus* and *S. epidermidis* (n=3 , ± SD).

5.3.2 *Identification of Main Compounds of L. cubeba and C. limon*

GC-MS chromatograph of litsea EO revealed 3 characteristic major peaks at retention times (RT) of 9.33 min, 17.70 min and 18.84 min (Figure 5.4). Lemon EO GC-MS chromatograph revealed a characteristic major peak at RT 9.59 min and also showed two minor peaks at RT of 17.55 min and 18.61 min (Figure 5.5).

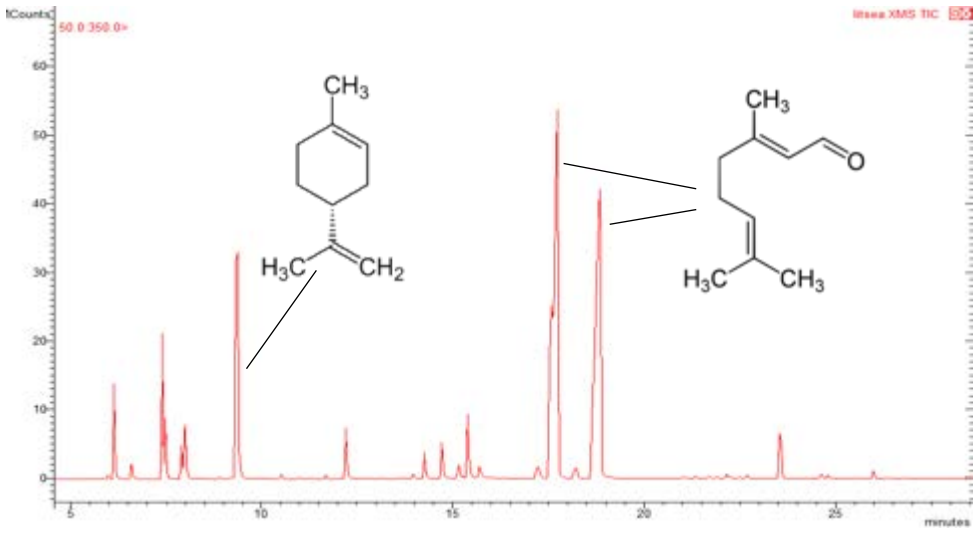


Figure 5.4 Gas chromatogram from GC-MS analysis of *L. cubeba* 1:100 in *n*-hexane showing major compound peaks at various retention times

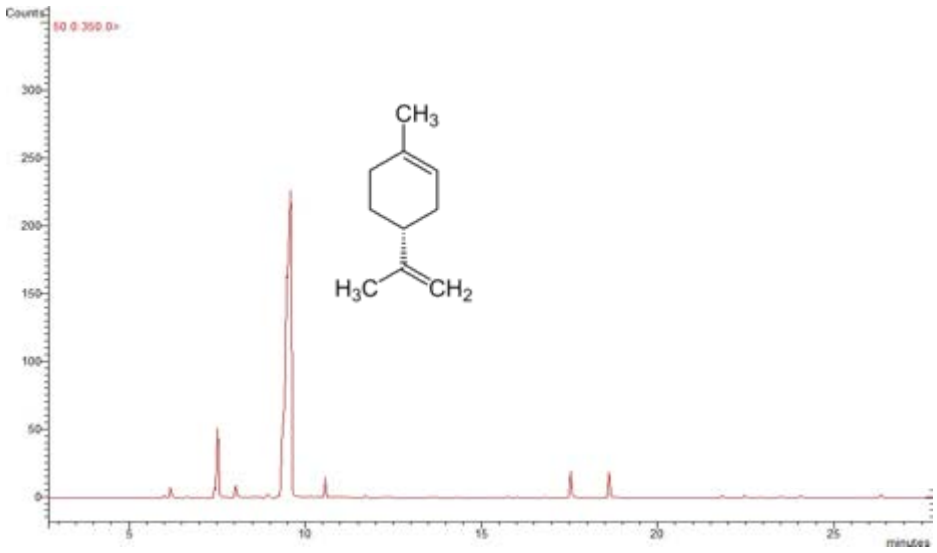


Figure 5.5 Gas chromatogram from GC-MS analysis of *C. limon* EO showing main peak of limonene compound

GC-MS analysis of pure reference standards confirmed the identity of the main peaks observed in the litsea EO and lemon EO (Figure 5.4 and Figure 5.5) as citral and limonene. Citral showed a m/z of 152.1 with two distinctive peaks at

RT 17.67 min and 18.61 min indicating the presence of both E/Z isomers ; limonene in turn showed a m/z of 136.1 at RT 9.55 min (Figure 5.6).

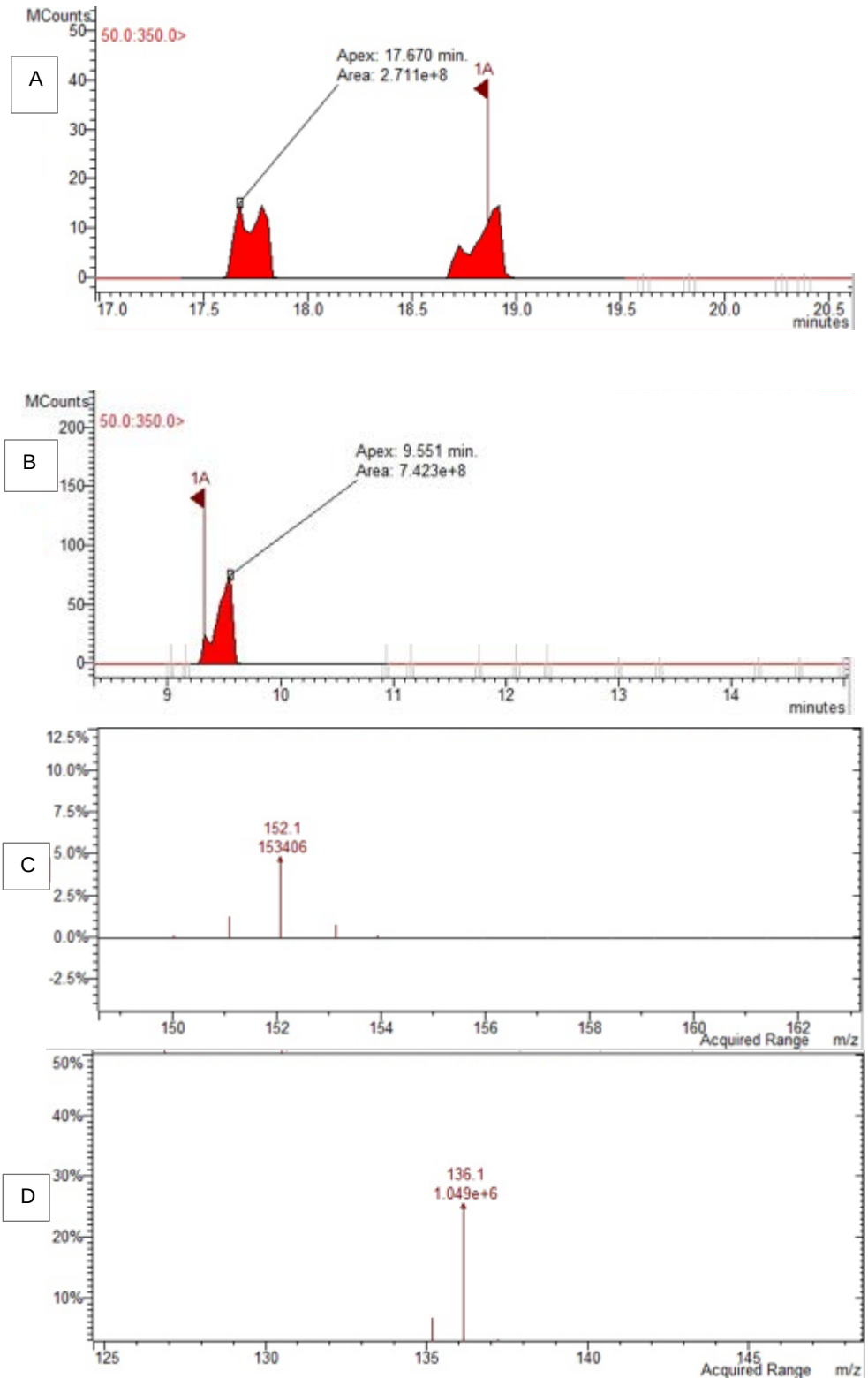


Figure 5.6 Gas chromatogram from GC-MS analysis of a pure standard of citral (A) pure limonene (B) and corresponding mass spectra for citral (C) and limonene (D).

These two compounds (citral and limonene) were subsequently used as markers for the quantification of the EOs within the emulsion formulations during storage stability studies and in-vitro release studies. Standard curves were created for both compounds at concentrations 0.78125 – 200 mM which can be seen in Figure **5.7** and Figure 5.8. Linear equations for citral and limonene are shown in Equations (9) and (10).

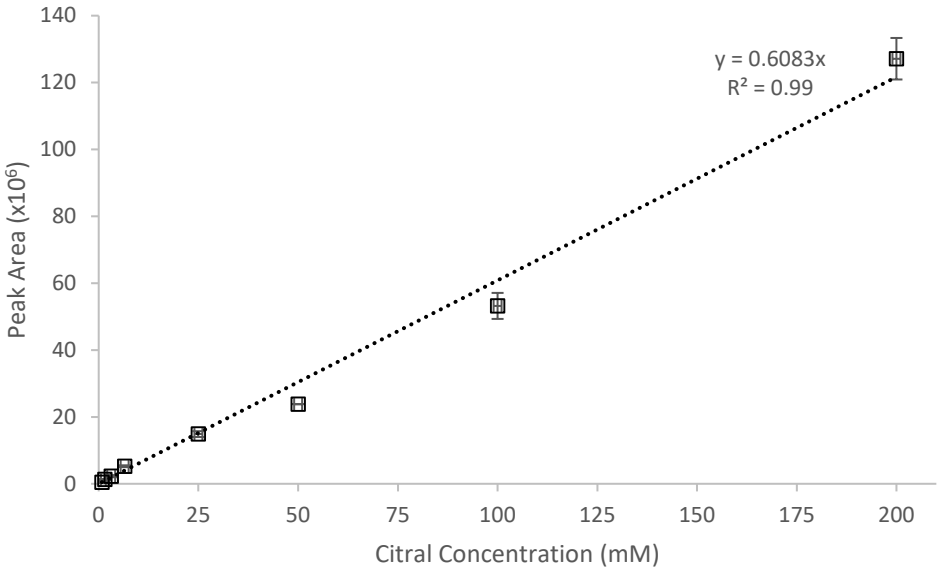


Figure 5.7 Standard curve of citral concentrations 0.78125 – 200 mM

Linear equation: $y = 0.6083x$, therefore $x = \frac{y}{0.6083}$ (9)

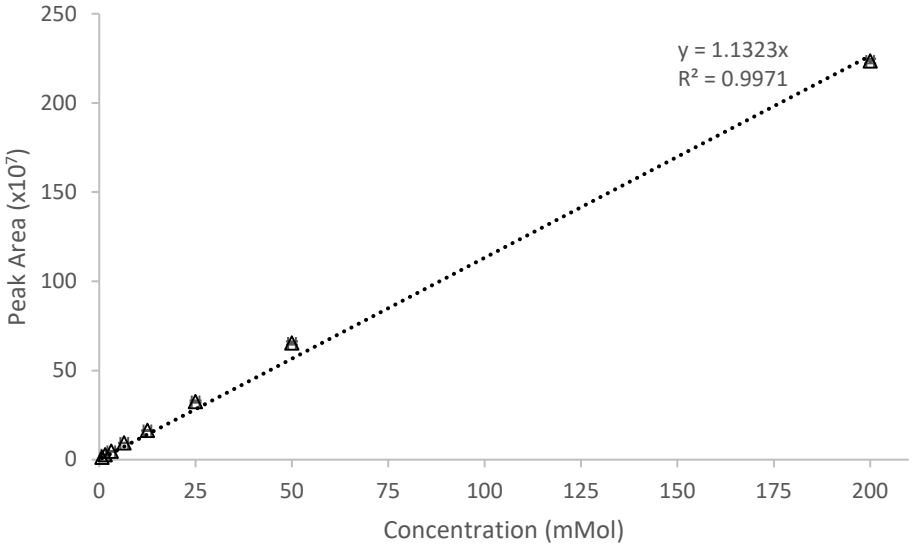


Figure 5.8 Standard curve of limonene concentrations 0.78125 - 200 mM

Linear equation: $y = 1.1323x$, therefore $x = \frac{y}{1.1323}$ (10)

5.3.3 *Rheology*

The viscosity profile of polymer solutions of 1% w/v chitosan (Figure 5.9) shows that the polymer solution exhibits shear thickening a non-Newtonian behaviour where there is an increase in viscosity with increased shear rate, typical of non-Newtonian pseudoplastic behaviour. At 20°C, the viscosity values of 1% w/v chitosan solution were 0.60 Pa. s at 0.05 s^{-1} and 2.19 Pa. s at 50 s^{-1}.

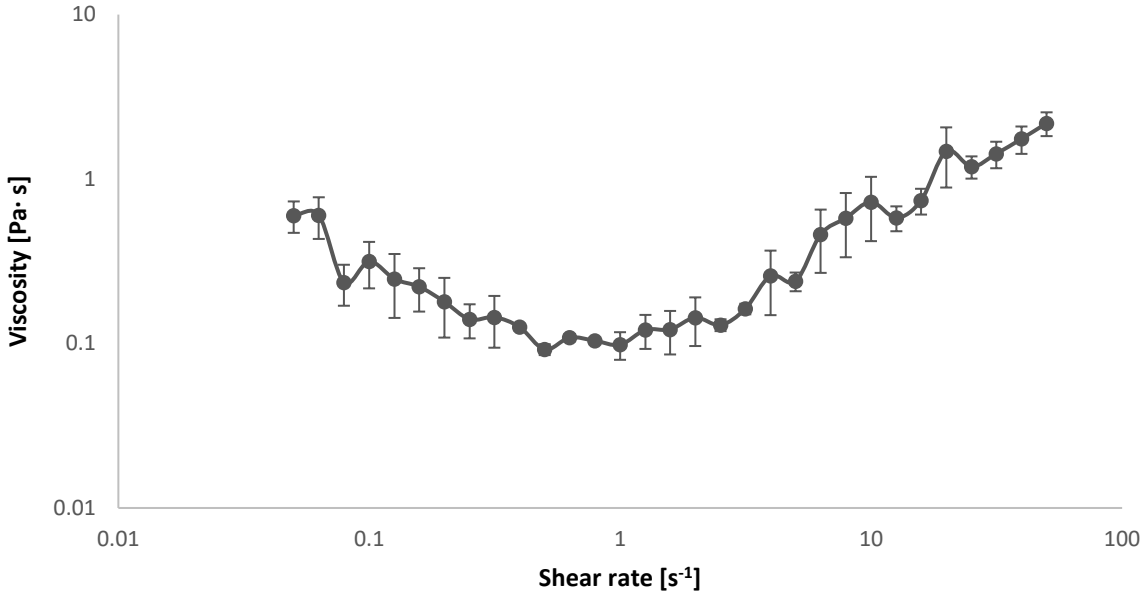


Figure 5.9 Influence of shear rate on the rheological curve of a 1% w/v chitosan solution at 20 ˚C

Polymer solutions of 1% w/v sodium alginate (Figure 5.10) also showed shear thinning with a decrease in viscosity as shear rate increased. The viscosity of the solution at 20°C was 25.75 Pa. s at 0.05 s^{-1} and 0.38 Pa. s at 10 s^{-1}; after which the viscosity increased again being 3.46 Pa. s at 50 s^{-1}.

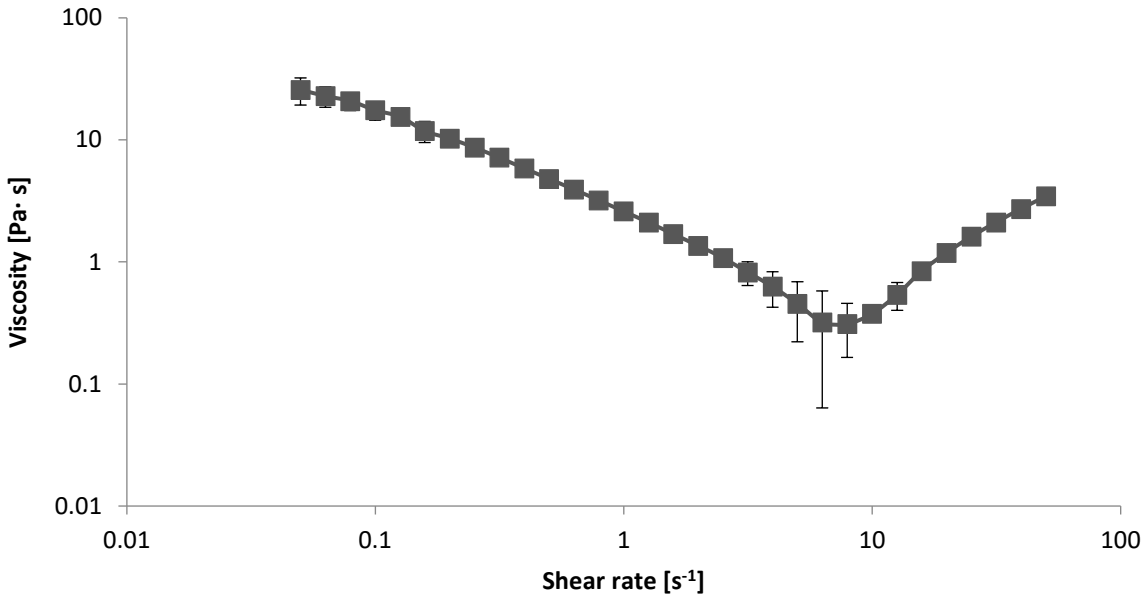


Figure 5.10 Influence of shear rate on the rheological curve of a 1% w/v sodium alginate solution at 20 ˚C

The flow behaviour of the emulsions containing 0.5% CS, 0.75% CS, and 1.0% CS was also analysed at two different strains 0.3% and 1% (Figure 5.11). Pseudoplastic behaviour was observed by all the tested emulsions, with a decrease in viscosity as shear rate increased, at both strain values tested. Generally, higher viscosity values (58.65 Pa. s and 34 Pa. s at shear rate of 0.05 s^{-1}) were observed with B1 (1% CS) emulsions when tested at strain of 0.3% and 1% respectively.

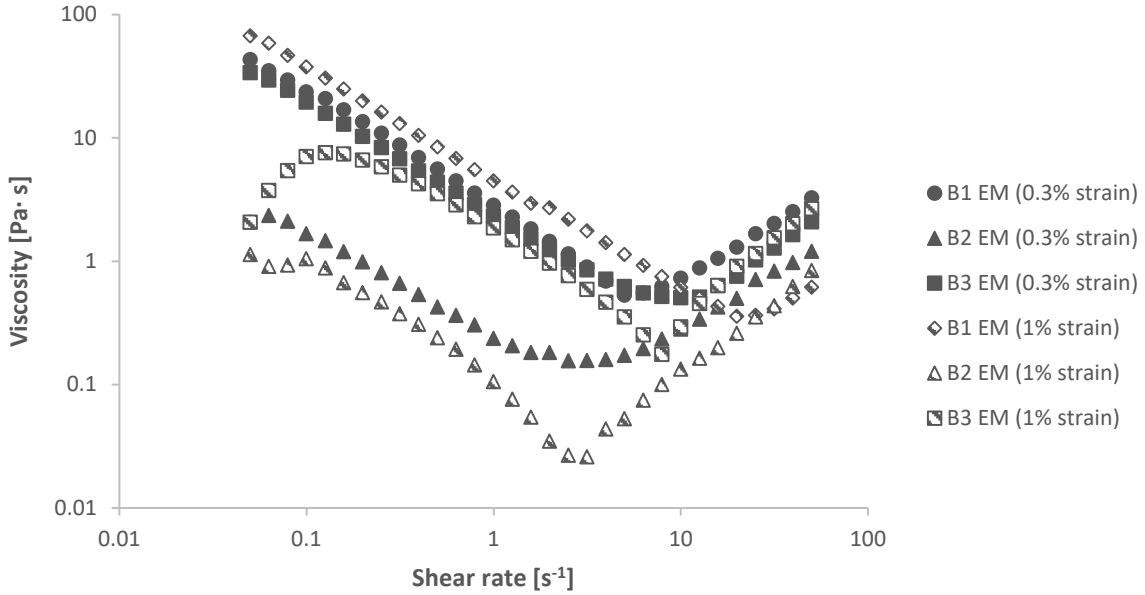


Figure 5.11 Influence of strain and shear rate on the rheological curve of emulsions containing 1.0 % chitosan (B1), 0.75% chitosan (B2) and 0.5% chitosan (B3) at 20°C

5.3.4 *Particle Size, Zeta Potential and Optical Microscopy*

The zeta potential (or ζ-potential) is a parameter commonly used to determine the electrostatic contribution to an emulsion's stability. The results (Table 5.2) showed slightly negative values of ζ-potential for all three concentrations of CS; the most negative value was seen with 1% chitosan emulsion at -8.16 mV and the highest with 0.5% chitosan at -3.75 mV. The DLS results showed that the emulsions tested did not show a significant difference ($p \geq 0.05$) in particle size between the emulsions of different chitosan concentrations. The average diameter for 0.5% CS, 0.75% CS and 1.0% CS were 1.146 µm, 1.398 µm and 1.556 µm respectively (Table 5.2). There was no significant

increase ($p \geq 0.05$) in the PI related to the concentration of chitosan within the emulsions (Table 5.2). The shape of the particles for each emulsion (0.5 – 1% CS) can be observed in Figure 5.11.

Table 5.2 Polydispersity index, particle size measurement and zeta potential measurement of litsea-lemon EO o/w emulsions containing 0.5-1% w/v chitosan (± SD).

Chitosan Concentration (%w/v)	Particle size (µm) ± SD	Polydispersity Index ± SD	ζ Potential (mV) ± SD
0.05	1.146 ± 0.114	0.514 ± 0.025	-3.745 ± 0.078
0.75	1.398 ± 0.293	0.495 ± 0.033	-5.290 ± 0.168
1.00	1.556 ± 0.142	0.500 ± 0.02	-8.160 ± 1.344

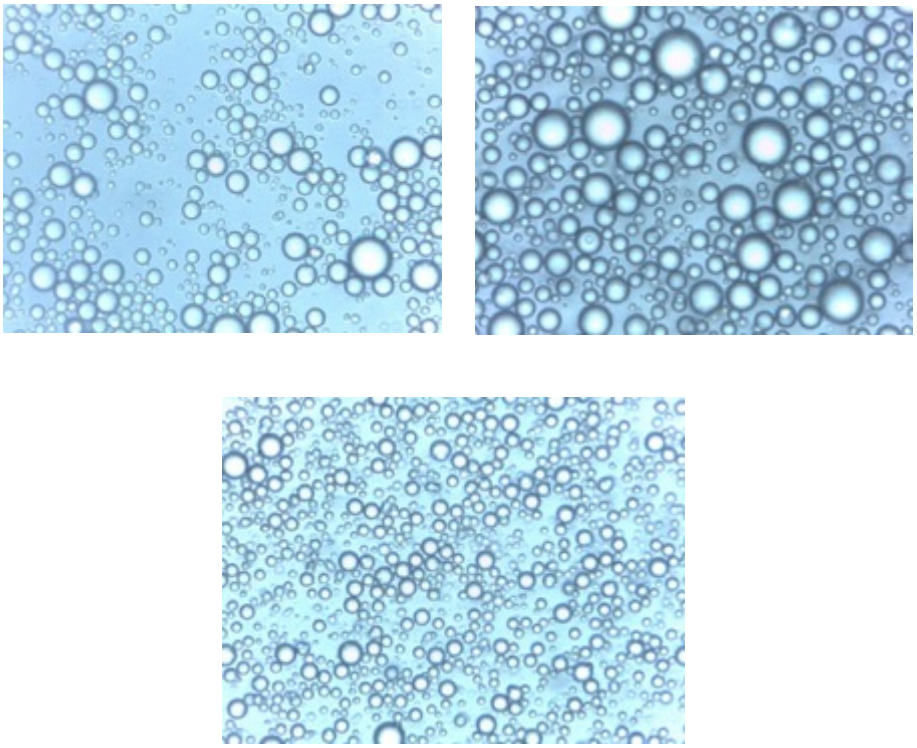


Figure 5.12 Optical microscope photographs from a series of 30% litsea-lemon EO o/w emulsions of varying chitosan concentrations: (a) 0.5%; (b) 0.75%; (c) 1.0% after 24 h of storage at ambient temperature.

5.3.5 *FTIR spectroscopy*

FTIR spectroscopy is a method commonly used to examine the structure of polymers, EOs and compounds, and the interaction of the molecules involved. In the FT-IR spectra for lemon (Figure 5.13) the most characteristic absorptions that could be observed were present at 2917.17 cm^{-1}, 1643.96 cm^{-1}, 1435.74. cm^{-1}, 1376.25 cm^{-1}, 885.51 cm^{-1} and 796.95 cm^{-1}. Specifically, the peak at 2917.17 cm^{-1} is due to the aromatic C-H stretching vibration, while the peaks at 1435.74. cm^{-1} and 1376.25 cm^{-1} could be attributed to the C-C stretch of the aromatic ring of limonene. The IR spectrum of litsea EO (Figure 5.13) was very similar to that

of lemon EO with characteristic peaks for litsea found at 2917.12 cm^{-1}, 1672.42 cm^{-1}, 1439.97 cm^{-1}, 1376.55 cm^{-1}, 1194.05 cm^{-1} and 1120.13 cm^{-1}. The peak at 1672.42 cm^{-1} is very strong compared to lemon EO and can be attributed to the C=O double bond found in citral compound.

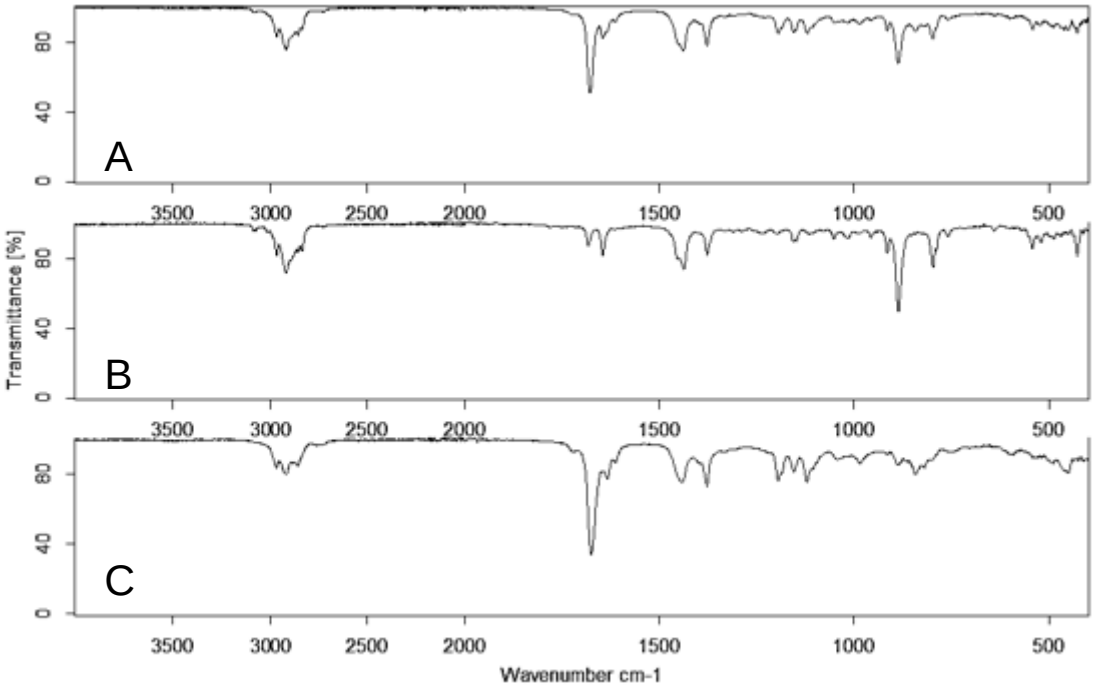


Figure 5.13 FTIR spectra of (A) litsea-lemon EO blend, (B) lemon EO and (C) litsea EO

The IR spectrum for limonene can be seen in (Figure 5.14) and the peak at 1643.96 cm-1 can be linked to the C=C double bond in the aromatic ring while the absorption of the ring C-H bending vibrations gave characteristic peaks at 885.51 cm-1. The IR spectrum of citral can also be seen in (Figure 5.14) and similar to that found in the spectrum for litsea a strong C=O bond peak can be seen at 1670.75 cm-1.

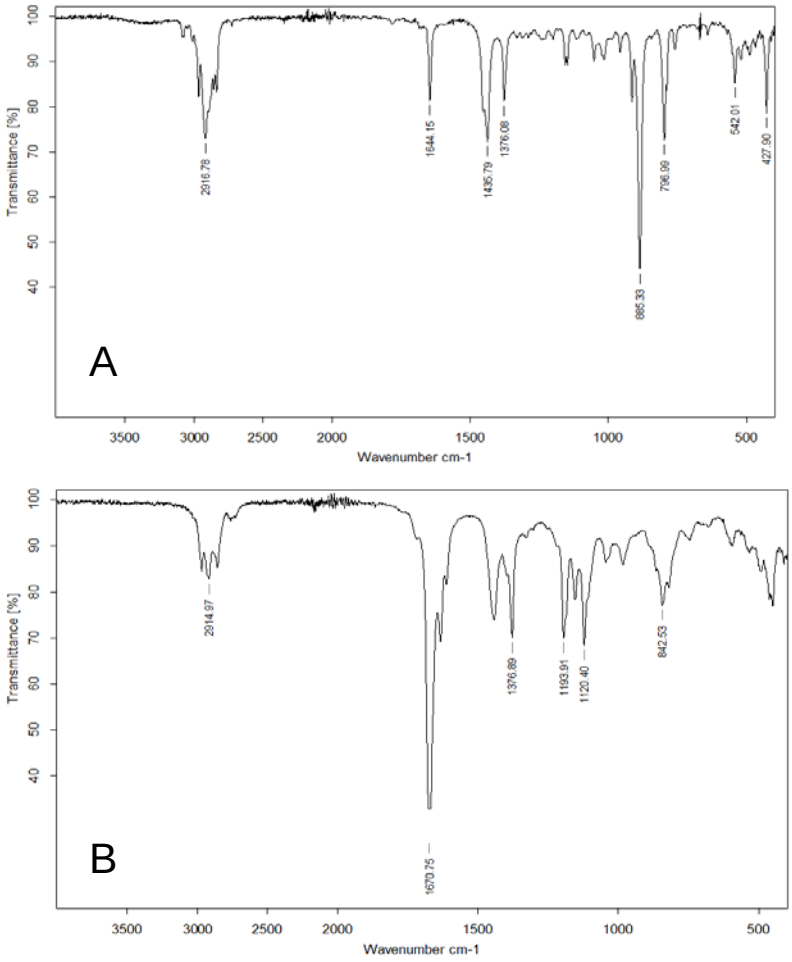


Figure 5.14 FTIR spectrum of a pure standards of limonene (A) and citral (B)

The IR spectrum of chitosan (Figure 5.15) showed a broad peak at 3295.15 cm^{-1} and at 1021.21 cm^{-1}. The broad peak appearing at 3295.15 cm^{-1} is due to stretching vibrations of O-H bonds while the peak at 1021.21 cm^{-1} corresponds to the C-O stretching bands. The spectrum for sodium alginate (Figure 5.15) showed characteristic peaks at 3228.11 cm^{-1}, 1591.83 cm^{-1}, 1406.09 cm^{-1} and 1023.39 cm^{-1}. These peaks found at 3228.11 cm^{-1}, can be attributed to the O-H stretching, those at 1591.83 cm^{-1} and 1406.09 cm^{-1} to COO- asymmetric and symmetric stretching. The peak at 1023.39 cm^{-1} can be attributed

to guluronic units. When the physical mixture of chitosan and sodium alginate was analysed (Figure 5.15), the resulting spectrum showed only two broad peaks, the first at 3363.88 cm^{-1} corresponding to hydroxyl (O-H) groups and second peak at 1623.99 cm^{-1} corresponding to a carbonyl (C=O) group; the peak at the region of 1021.21- 1023.39 cm^{-1} showed a large decrease in intensity.

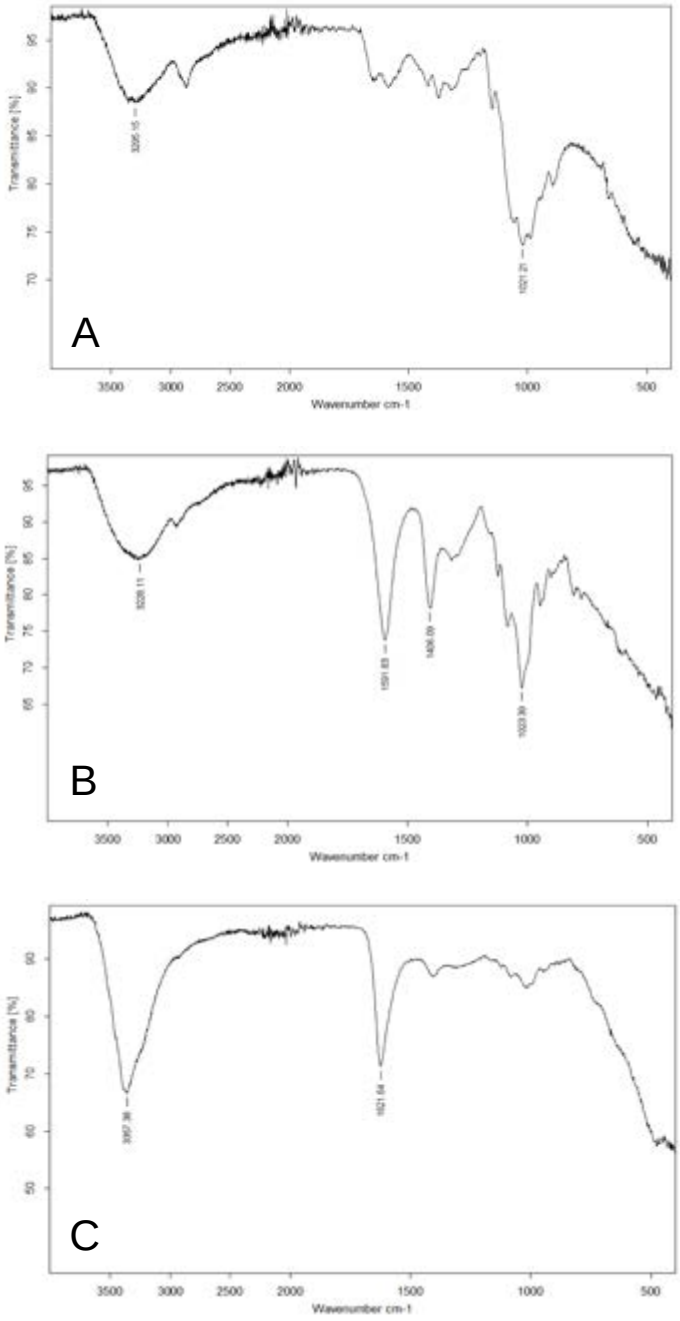


Figure 5.15 FTIR spectra of (A) CS powder, (B) SA and (C) a blend of CS and SA (1:1 ratio)

When the emulsions with chitosan concentrations of 0.5 -1% w/v were analysed by FTIR the resulting spectra showed the same characteristic two peaks seen in the spectrum for chitosan/sodium alginate physical mixture (Figure 5.16) any other characteristic peaks seen in the spectra of individual components disappeared or became more prominent possibly due to interactions of the chitosan and sodium alginate.

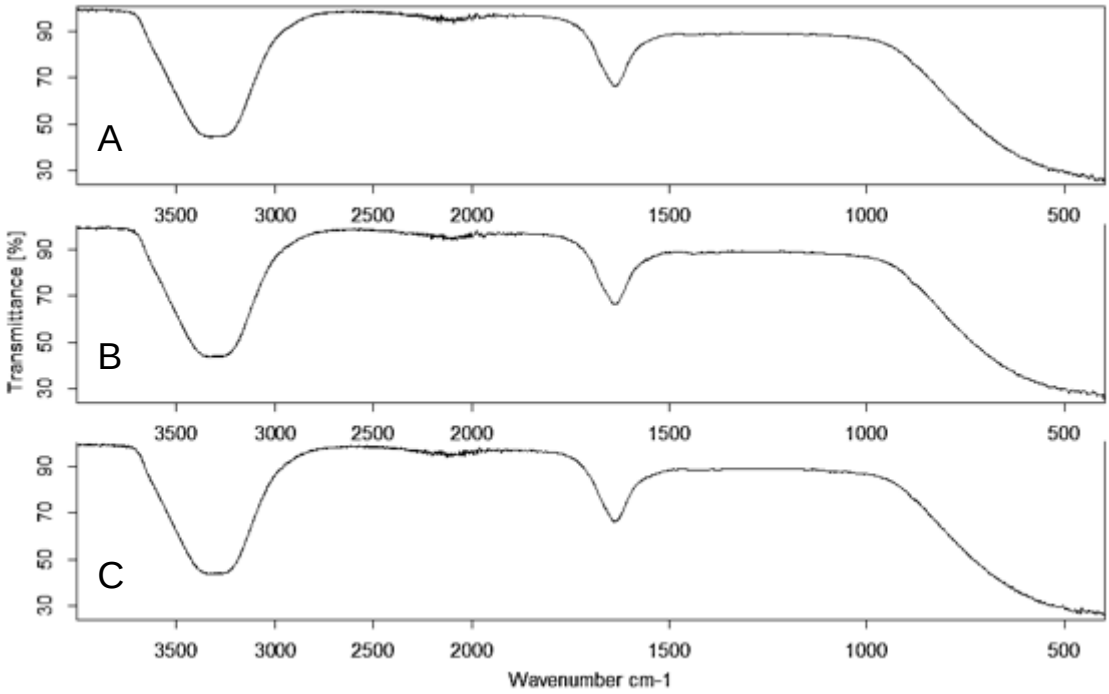


Figure 5.16 FTIR spectra for 30% litsea-lemon EO emulsions of varying concentrations of chitosan (A) 0.5% w/v, (B) 0.75% w/v and (C) 1% w/v.

5.3.6 *Thermal Analysis by Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA)*

Thermal analysis was conducted on pure litsea and lemon EOs, reference standards of citral and limonene, the polymers of chitosan and sodium alginate and the emulsions. The TGA plots showed onset thermal transition points of 62.17°C for formulation B1, 68.71°C for B2 and 64.04°C for B3, while their boiling points were observed to be 134.95°C, 136.64°C and 117.28°C respectively (Figure 5.17 a).

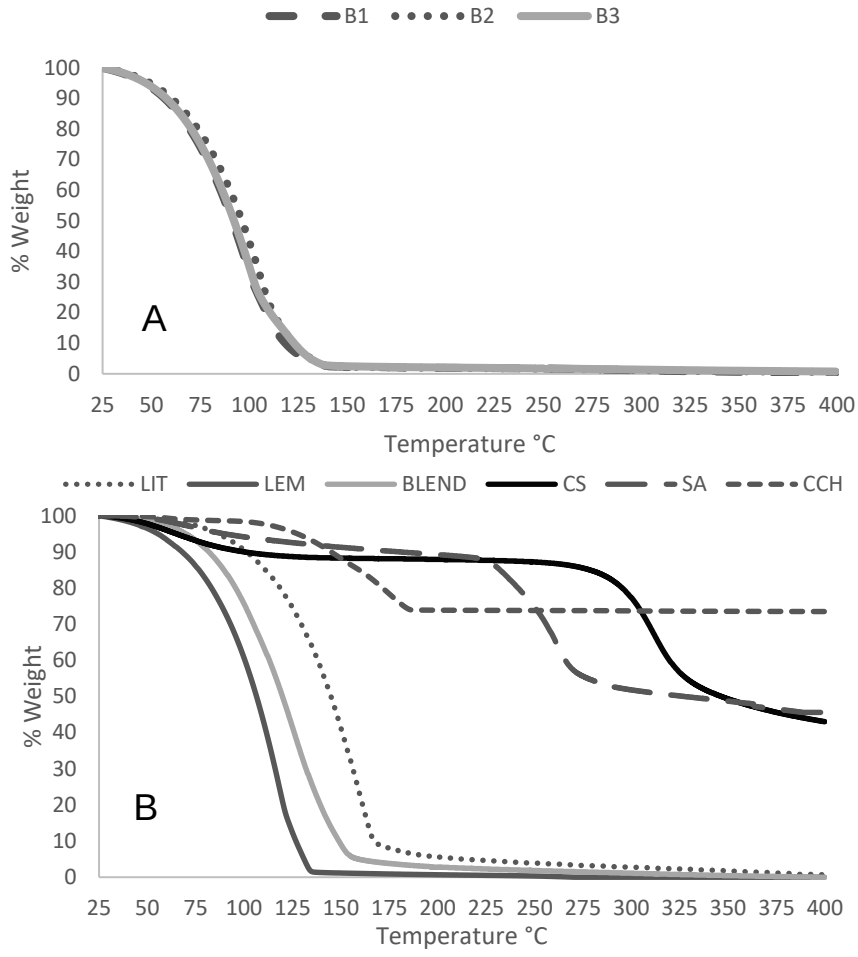


Figure 5.17 TGA Thermograms for (A) emulsion formulations B1, B2 and B3 and (B) emulsion raw components litsea EO (LIT), lemon EO (LEM), 1:2 litsea and lemon EO blend (BLEND), chitosan (CS), sodium alginate (SA), calcium

The thermal transitions seen in the TGA graphs for litsea and lemon shows that their weight loss onset is 118.14°C and 83.98°C respectively (Figure 5.17 b) and when a physical mixture (blend) of the two oils was analysed, the onset of the blend was 86.58°C (Figure 5.17 b).

The DSC profile for raw CS was characterised by a large endothermic event at 126.99°C and a large exothermic peak at 317.73°C (Figure 5.18 b). The thermogram for SA showed a broad endothermic peak from 97.07°C to 170.80°C and another broad exothermic peak from 209.02°C to 276.21°C, while CCH showed a small endothermic event at 54.22°C and three clustered endothermic peaks with the largest being at 211.41°C (Figure 5.18 b). When a physical mixture of CS, SA and CCH was analysed, the resulting thermogram showed a sharp endothermic event at 50.75°C, (also observed in CCH) and a broad endothermic event at 158.37°C (also observed in SA and CS thermograms); the exothermic peak observed in the thermogram of CS was no longer present (Figure 5.18 b). Thermograms for individual oils showed a sharp endothermic peak at 228.50°C for lemon EO while a similar endothermic peak was observed at 232.93°C for litsea EO; when a blend of the EOs was analysed these sharp peaks were no longer present, with the only a small broad peak observable at 66.42°C (Figure 5.18 c).

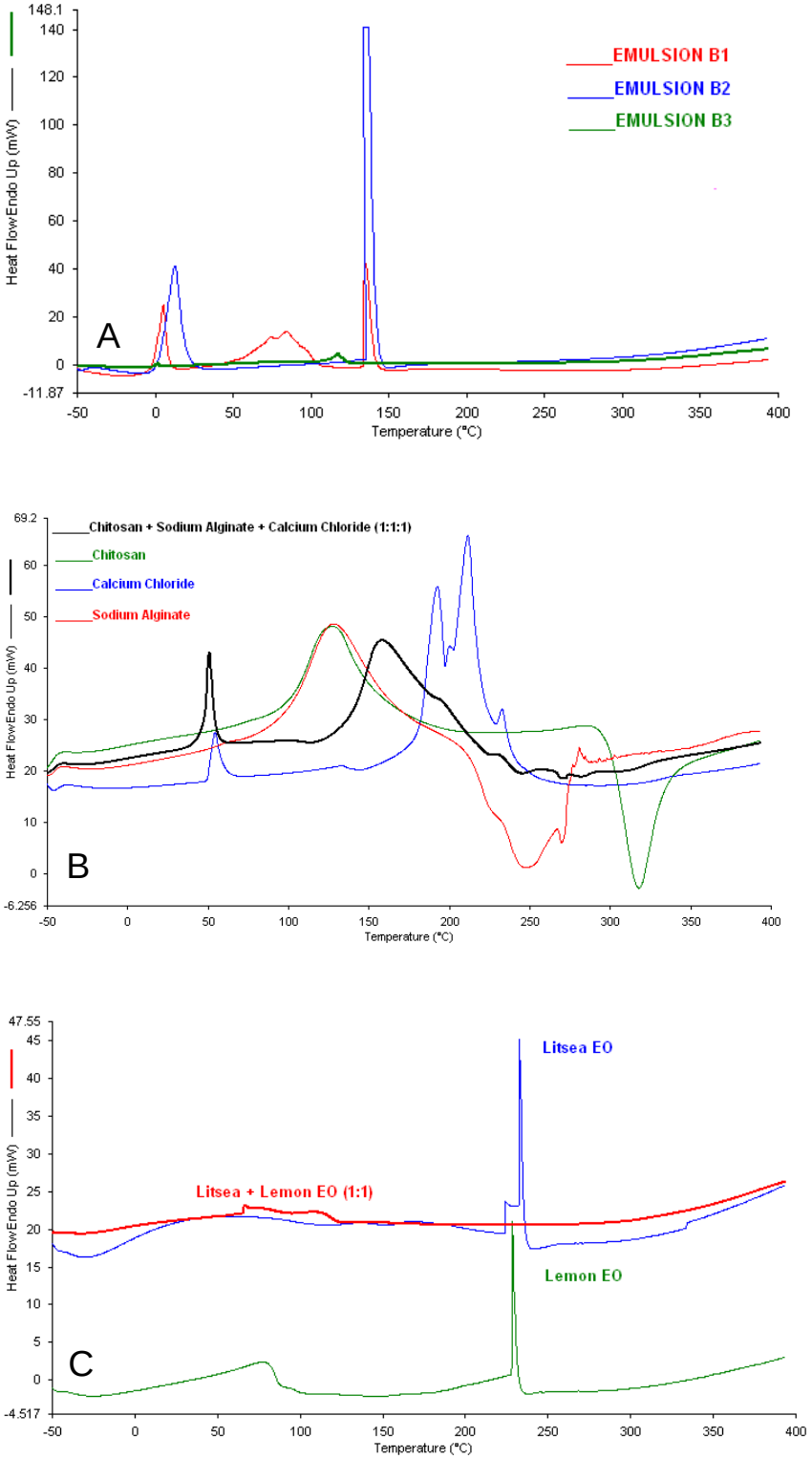


Figure 5.18 DSC thermogram for (A) emulsion formulations B1, B2 and B3; (B) for raw solid components of emulsions chitosan, calcium chloride and sodium alginate and a physical mixture of each; and (C) litsea and lemon EOs and their blend.

The DSC profiles for all three emulsion formulations revealed sharp endothermic peaks at 134.95°C and 136.64°C for formulation B1 and B2 respectively; B1 formulation also showed a smaller broad peak between 58.75°C and 104.84°C before this sharp peak (Figure 5.18 a). A much smaller endothermic peak was observed for formulation B3 at a lower temperature of 117.28°C (Figure 5.18 a).

5.3.7 *Release Studies*

Results on the investigation of the release of the main compounds (citral and limonene) of the encapsulated litsea and lemon EOs showed that the emulsions of 1% CS are characterised by a large burst effect as a rapid release of citral is observed (Figure 5.19) and limonene (Figure 5.20) is observed within 10 min of the release test for emulsions of 1% CS (B1), with 70.11% of limonene released. Formulations B2 and B3 demonstrated a much smaller burst effect for citral and limonene (Figure 5.19 and Figure 5.20). Limonene in formulation B1 was completely released within 30 min, while in formulations with lower CS concentration B2 and B3 only 21.66 % and 15.53 % of limonene respectively was released (Figure 5.20). In comparison, after 30 min, only 1.68% and 1.21% of citral was released from B2 and B3 formulations respectively (Figure 5.19).

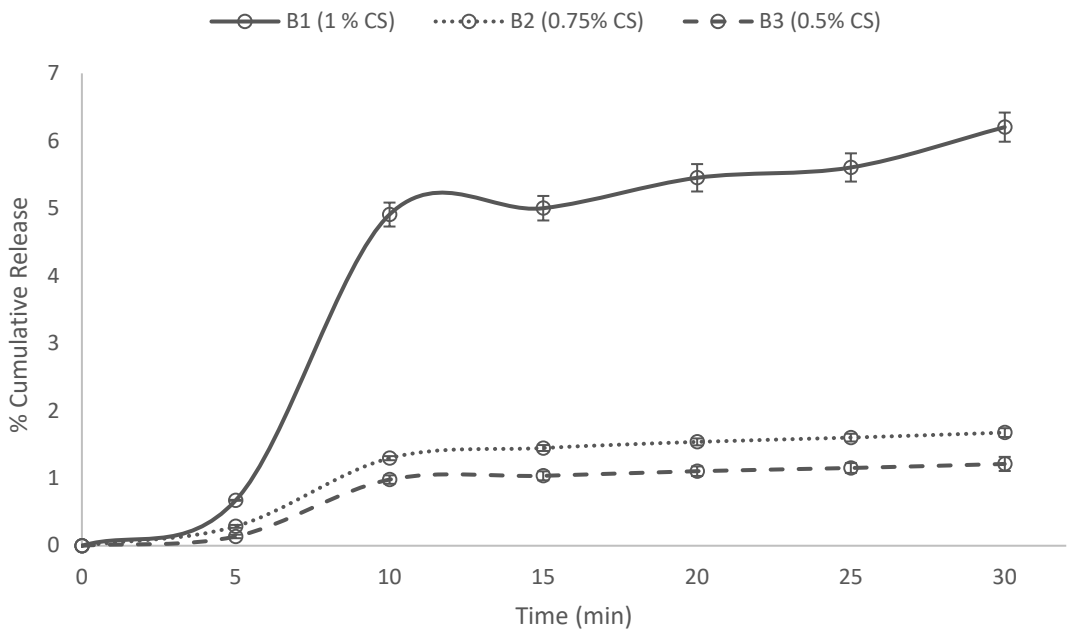


Figure 5.19 Release of citral compound from a litsea-lemon EO O/W emulsions of 0.5-1% CS concentration over 50 min in acetate buffer (pH 5) with 2% Tween 80 at 32°C

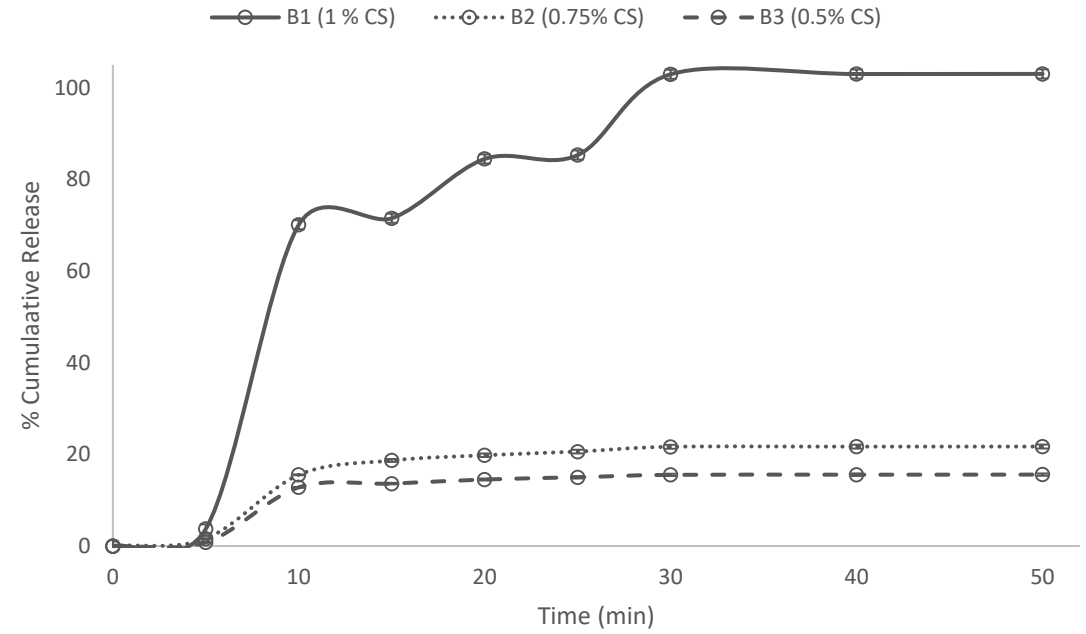


Figure 5.20 Release of limonene compound from a litsea-lemon EO o/w emulsions of 0.5-1% CS concentration over 50 min in acetate buffer (pH 5) with 2% Tween 80 at 32°C

5.3.8 *Physical Stability of Litsea-Lemon EO O/W Emulsions*

5.3.8.1 Creaming and Accelerated Creaming Stability

The results of this study showed that when the litsea-lemon EO o/w emulsions without surfactants were stored over a period of 5 weeks, the % CI for emulsions with 0.75% w/v CS and 1% w/v CS showed very similar trends with % CI of 13.33% and 12.66% respectively after 14 days of storage (Figure 5.21). Emulsions with 0.5% w/v CS exhibited low stability, with an average CI of 54.67% recorded by day 5.

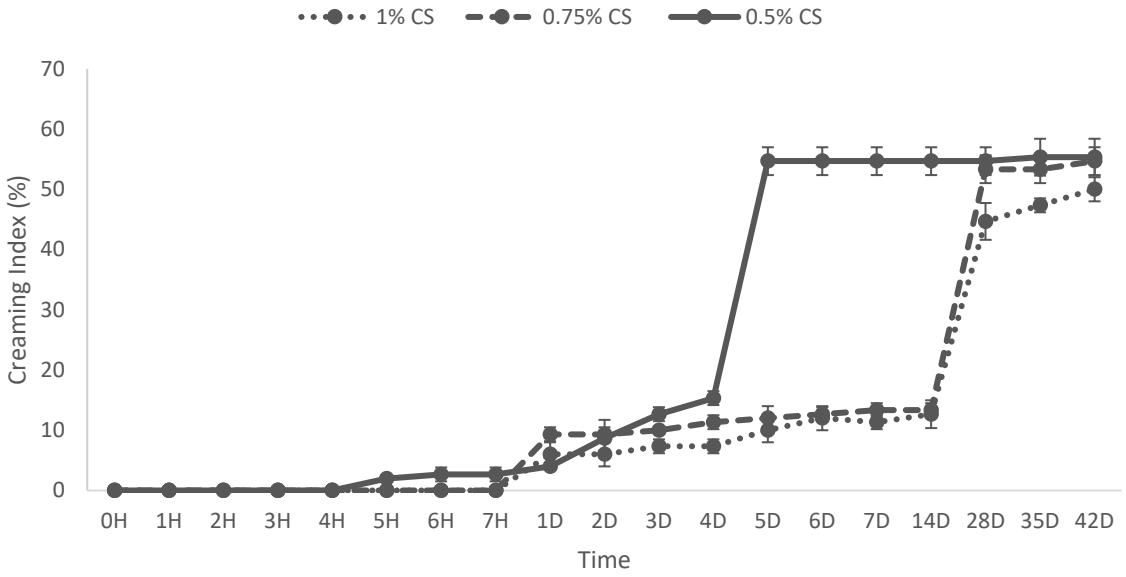


Figure 5.21 % CI of 30% litsea/lemon o/w emulsions at different chitosan concentrations (0.5 – 1% w/v) over 5 weeks of storage at ambient temperature (H= hours; D= days)

When the emulsions were subjected to centrifugal force to predict the long term stability of the emulsion (months) it was seen that all emulsions showed

low stability to the applied stress as over 60% creaming index was recorded for all 3 emulsions within 3 cycles (15 min) of centrifugation (Figure 5.22).

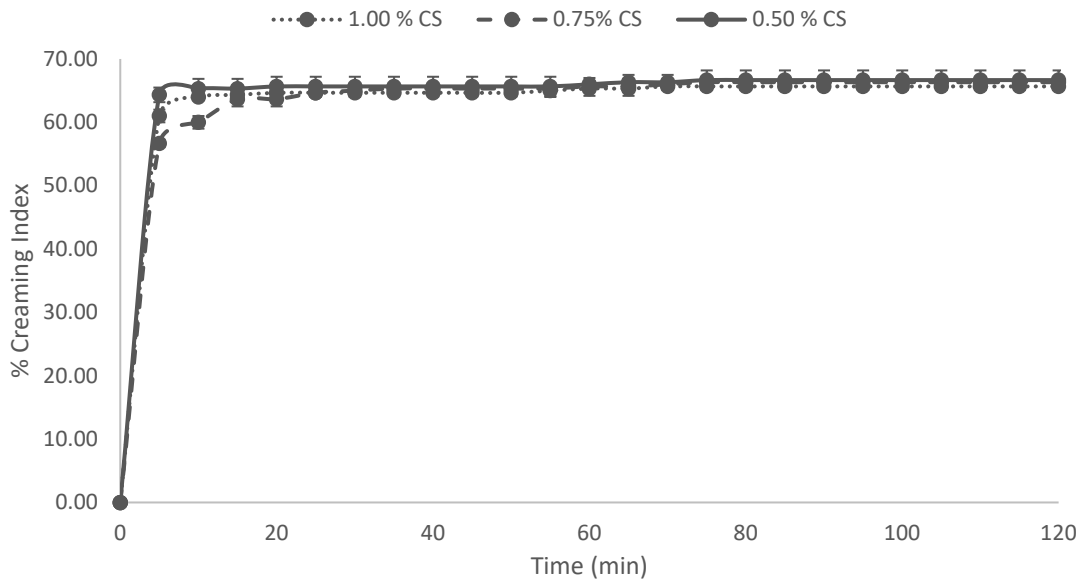


Figure 5.22 % CI over time of 30% EO emulsions at different CS concentrations after 2 hours of centrifugation

5.3.9 *Chemical Stability During Storage*

The chemical stability of the emulsions over time was also assessed by comparing the quantified amount of citral present within emulsion with 1.0 % CS (B1), 0.75% CS (B2) and 0.5% CS (B3) within 28 days, stored at 15°C and at 40°C (Figure 5.23). Large fluctuations in concentrations of limonene and citral were observed, only results of citral concentration from 0 hour (time 0) and 28 days are shown; the results in Figure 5.23 show that the presence of citral in formulation B1 when stored at 15°C significantly decreased ($p \leq 0.05$) from 136.81 mM to 0.02 mM, while when stored at 40°C there was an increase in citral concentration after 28 days from 114.33 mM to 254.23 mM. There was a small

decrease (13.82 mM) in citral concentration after 28 days for formulation B2 stored at 15°C, but a large decrease when it was stored at 40°C from 222.85 mM to 20.23 mM. The presence of citral within formulation B3 significantly increased ($p \leq 0.05$) when stored at 15°C going from 224.67 mM to 482.08 mM, while when stored at 40°C it decreased to 76.51 mM (Figure 5.23).

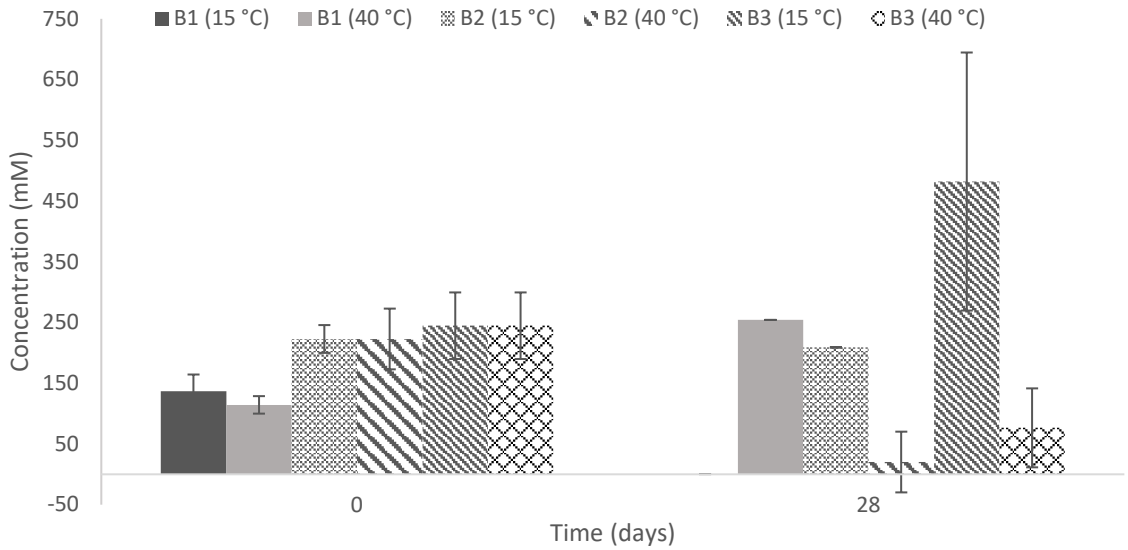


Figure 5.23 Changes in concentration of citral in emulsion formulations B1, B2 and B3 stored at 15°C and 40°C for 28 days

5.4 Discussion

All the emulsions tested showed a non- Newtonian behaviour where there was a decrease in viscosity when shear rate was increased. The concentration of CS influenced emulsion viscosity, and it was seen that a higher concentration lead to higher viscosity (Figure 5.11). A reduction in particle mean diameter was observed with decreased amount of CS in the formulation (Table 5.2); it is imaginable that due to the higher viscosity of formulations with higher CS concentrations the homogenisation process was able to better reduce the particle size of a less viscous emulsion. A fixed homogenization pressure was used and Garcia et al (2012) reported a linear relationship between particle mean diameter and emulsion viscosity in their studies (Garcia et al., 2012). The average particle size of formulations B1, B2 and B3 was 1.556 µm, 1.398 µm and 1.146 µm respectively with a nearly neutral zeta potential, which may be attributable to the low pH (less than 5) of the emulsions (Table 5.2); Wasupalli and Verma (2018) recorded zeta potential measurements of -1.87 ± 0.87 mV for self-assembled complexes of sodium alginate and chitosan prepared at pH 5.5 but highly negative values (-23.67 ± 2.08 mV) when prepared at pH 8.5 (Wasupalli and Verma, 2018).

When the presence of citral and limonene within the emulsion formulations was assessed, results showed that higher concentration of citral could be extracted from each formulation compared to limonene, with average citral concentrations observed in B1, B2 and B3 ranging from 114.33-244 mmol/ml

while limonene concentrations ranged from 2.21-10.77 mmol/ml (results not shown). A complete release (100%) of limonene was observed in formulation B1 within 30 min (Figure 5.21) compared to 5.45 % of citral released from B1 after 30 min (Figure 5.19). A general trend could be observed as release of citral and limonene from all batches was characterised by an initial rapid burst release, followed by a slowed sustained release (Figure 5.19 and Figure 5.20). A similar profile was observed by Kotronia et al (2017) when assessing the in vitro release of oregano EO from a β-cyclodextrin complex; the profile was characterized by a burst effect within the first 1 hour and 45 min and then a slower release rate over 10 days (Kotronia et al., 2017). The release of tea tree EO from topical formulations has also been assessed by Sgorbini et al (2017) where the markers 1,8-cineole, 4-terpineol and α-terpineol maximum release from a 30% ointment were 32.2%, 10.1% and 7.7% within 50 hours (Sgorbini et al., 2017).

Limonene is prone to autoxidation when exposed to air for prolonged periods, forming hydroperoxides which can further break down into by-products such as carveol acetate and limonene oxide (Djordjevic et al., 2007; Kern et al., 2014). When the chemical stability of the emulsions was assessed at 15 ºC and 40 ºC within 28 days limonene could only be recovered at extremely low concentrations or not at all with fluctuating concentrations observed (results not shown); citral concentrations after 28 days varied with each formulation with nearly a 200% increase observed for formulation B1 when stored at 40 ºC but complete decrease (100%) when stored at 15 ºC (Figure 5.23). The irregular variation in the compounds at both studied temperatures could be attributed to

the nature of the compounds as changes in temperature, humidity, light can all affect their concentration; strategies to prevent degradation have been suggested, including reduction of temperature during storage and alteration of oxygen pressure, however, these methods may be impractical (Djordjevic et al., 2007; Sharma and Lee, 2016). As expected from storage stability studies, after 28 days, the emulsions experienced cracking (an irreversible separation of the oil phase) at both 15ºC and 40ºC indicating physical instability and were discoloured at 40 ºC indicating a reaction such as oxidation could have occurred. TGA is an important method to determine the degradation properties of formulations and individually assess their components; thermograms for B1, B2 and B3 showed that all 3 emulsions displayed almost identical profiles with Figure 5.17a showing significant weight loss (over 90%) between 62 -110 ºC for B1, 68 -113 ºC for B2 and 64- 112 ºC for B3. Though only 1 thermal event can be seen in the TGA thermograms, DSC analysis profiles, showed 2 endothermic events after 0 ºC for B1 which did not present in the TGA results (Figure 5.18).

Formulations with CS concentration ranging from 0.5 to 1% were carried forward for analysis due to their visual physical stability (no phase separation within 24 hours) and their antimicrobial activity against *S. aureus, E. coli* and *S. epidermidis.* The most sensitive bacterial strain was *E. coli* against all three formulations B1 (1% CS), B2 (0.75 % CS) and B3 (0.5 % CS) with the largest ZOI observed of 44.00 mm by B3 (Table 5.1). A study by Din et al (2014) on clove and cumin EOs emulsions, on the other hand, found the at *E. coli* was the least sensitive organism to cumin emulsion and *S. aureus* the most sensitive with a

ZOI of 16.6 mm for a 25% v/v cumin emulsion (Din et al., 2015). Oregano EO has also been encapsulated by emulsification and the system was stabilised by cellulose nanocrystals prepared from microcrystalline cellulose, its antimicrobial activity was evaluated against *E. coli* and *S. aureus* and found to have MICs of 12.5 µl/ml for both organism (Zhou et al., 2018). Many encapsulations of EOs by emulsification reported in literature have involved nanoemulsions; Lu et al (2018) developed a nanoemulsion with citral EO and found that *S. aureus* was the most susceptible organism tested with an average ZOI of 19.2 mm, whilst *E. coli* and *P. aeruginosa* gave zones of 9.4 mm and 6.2 mm respectively (Lu et al., 2018).

The main compounds identified in litsea and lemon EOs were limonene and citral (Figure 5.6). It has been reported that citral has the ability penetrate the lipid structure of bacterial cell walls causing denaturation of proteins and damage to the cell wall leading to death of the cell (Lu et al., 2018). When assessing the individual antimicrobial activity of the emulsion ingredients against *S. aureus, E. coli* and *S. epidermidis*, the EO blend (litsea and lemon EO, 1:2 ratio) and citral standard had similar activity with ZOIs range of 24.00 mm to 34.94 mm for the blend and a range of 25.00-30.05 for citral, compared to limonene standards which gave ZOIs between 6.97 – 18.54 mm (Figure 5.3). Both the EO blend and citral had greater activity against *E. coli* (Figure 5.3), this agrees with the results from the assessed antimicrobial activity of the emulsions which also found *E. coli* to be the most susceptible organism (Table 5.1).

The physical stability of the emulsions formulations developed in this present study renders them more suitable for a “fresh application” type of use; it was observed that emulsions with lower concentration of CS experienced poorer physical stability, with emulsions of 0.5% CS concentration giving a CI of 54.67% recorded by day 5 (Figure 5.21). Emulsions with higher CS concentration showed increased stability, indicating that the stability of the emulsion is reliant on the concentration of CS within the system. Creaming is an undesirable phenomenon in emulsions and generally occurs due to gravitational or centrifugal forces that lead to the agglomeration of droplets and therefore an increase in particle size as they accumulate at the top of system (Mcclements, 2007). According to McClements (2007) the stability of an emulsion to separation can be improved by reducing the droplet size. In this study emulsions samples with CS concentration above 0.5% did not exhibit any visible instability for the first 7 hours (Figure 5.21). Garcia et al (2012) found that a basil EO emulsion formulated with gum Arabic did not exhibit any creaming in the first 24 h (Garcia et al., 2012). Moschakis et al (2016) observed that a sunflower emulsion was stable and did not exhibit any creaming process over 7 days (Moschakis et al., 2016). Although creaming is an undesirable process, unlike cracking, it is a reversible process that can often be reversed by simple shaking which re-disperses the product, much like a suspension system, allowing for use of the formulation for its intended application (such as textile padding) for a period of time. Emulsion formulations based on EOs, which are prone to changes in composition and chemical degradation, therefore could be tailored to fresh application (using the formulation within 24 hours of production) such as the treatment of textiles which could then be

packaged in ways that protect the product from environmental conditions that can lead to degradation.

In conclusion, the microencapsulation via emulsification of plant EOs only using crosslinked chitosan and sodium alginate is a toxic free method which excludes toxic chemicals and is based on only natural products. Natural chitosan-sodium alginate emulsions encapsulating a synergistic antimicrobial blend of litsea and lemon EOs were formulated and the resulting emulsion provides an eco-friendly antimicrobial finishing for the treatment of textiles.

Chapter 6. Treatment of Cotton and Polyester Textiles with Microencapsulated Antimicrobial Litsea-Lemon EO Blend

6.1 Introduction

The development of antimicrobial textiles has become an active and important research area, with a variety of possible applications and functions for antimicrobial textiles being explored for their ability to address many problems, ranging from preventing spread of infectious diseases (more importantly those that are drug-resistant) to addressing issues like body odour on sportswear attire. Textiles can be favourable environments for bacteria, fungi and moulds due to their large surface area and humidity, warmth and a high source of nutrients making them desirable for their growth of microorganisms (Ibrahim and Abd El-Salam, 2015).

Antimicrobial finishes are applied to textiles for the purpose of (1) controlling the spread of disease, (2) preventing odour caused by perspiration, stains and soiling on fabric and (3) controlling damage of fabric caused by decomposition (Yip and Luk, 2016). Textiles that inhibit the growth of microorganisms are called biostatic functional textiles, they control the growth and spread of bacteria (bacteriostats) and fungi (fungistats) while those that kill bacteria and fungi are called biocidal textiles (bactericides and fungicides). Biostatic textiles may be more tailored to controlling body odour on sportswear or in the preservation of textile items, while biocidal textiles will be more suited to

medical applications and providing antimicrobial protection to humans by eliminating pathogens completely and quickly upon contact (Sun, 2016).

The use of effective antimicrobial agents that are safe**,** biodegradable, environmentally non-toxic, cost-effective and with selective activity towards microbes (without causing microbial resistance) is now in higher demand as consumer preference is becoming increasingly geared towards natural antimicrobials. However, textiles treated with natural and plant-based agents are still in the development stage whilst commercially available antimicrobial textiles are treated by synthetic compounds like triclosan, QAC, silver and polybiguanides (Zhao et al., 2016). Studies have reported good activity by triclosan, with an MIC_{50} (MIC required to inhibit 50% of isolates) of 0.12 µg/ml observed against *S. aureus* clinical isolates (Schmid and Kaplan, 2004). The halogenated-phenol triclosan is regularly used as a textile finish in the production of antimicrobial socks and household textiles, with triclosan coated textiles showing good antibacterial activity even after 50 washes (Orhan et al., 2007). Similarly, Ranganath and Sarkar (2014) observed that after 50 washes, a triclosan treated polyester-cotton fabric blend maintained activity, with 100% reduction of *S. aureus* and *E. coli* reported (Ranganath and Sarkar, 2014). Dhiman and Chakraborty (2015) evaluated the antimicrobial activity of triclosan finished cotton against *S. aureus* and *E. coli* with 100% reduction observed against both organisms; after 5 washes antimicrobial activity was retained with a reduction of >98% observed for both *S. aureus* and *E.* coli (Dhiman and Chakraborty, 2015). QAC treatments have also been reported as durable with a

study on QAC treated cotton and polyester garments reporting maintained activity after 10 washes against *S. aureus* and *E. coli* (Saad and Gabr, 2015).

However, synthetic compounds that have been used to provide durable antibacterial activity on textiles, have been found to have a toxic effect on humans and the environment, and have raised concerns over bacterial resistance, allergies and side effects for users (Alihosseini, 2016; Hilgenberg et al., 2016). There have been various reports of bacterial resistance to triclosan and health concerns have been raised on its potential to disrupt the thyroid in humans, as well as environmental concerns due to toxic products formed during degradation and thus the use of triclosan as an antimicrobial for textiles has reduced (Dann and Hontela, 2011; Gao and Cranston, 2008; Yazdankhah et al., 2006).

Due to their bioactivity, low toxicity and biocompatibility, natural products such as EOs, have gained increased interest for both medical and cosmetic textiles, with a growing demand for textiles that provide antimicrobial ability, insecticidal properties, pleasant odours, UV protection and the promotion of health and fitness (Alihosseini, 2016). The main challenges faced with the application of natural products like EOs is their durability, shelf-life and antimicrobial efficiency which is why further research must be carried out to evaluate their use in the formation of bioactive textiles (Ali et al., 2014). Other methods involve, chemical modification of the fabric fibres, coating, spraying, grafting and microencapsulation. Microcapsules can be applied to the textile fibres using the pad-dry-cure method, also used by Dhiman and Chakraborty (2015) to develop triclosan and cotton finished cotton; spraying, impregnation or

screen printing-techniques can also be used (Ali et al., 2014; Dhiman and Chakraborty, 2015). The pad-dry-cure method is also used to impart mosquito repellency to fabric; EOs like citronella have been microencapsulated and applied using the pad-dry-cure method to confer mosquito repellent properties to cotton fabric (Specos et al., 2010).

Test methods commonly used to test antimicrobial textiles finishes include the parallel streak method (AATC 147), this is an inexpensive and quick method to determine whether a fabric sample displays antimicrobial activity. In this method, samples are laid on inoculated agar plates in parallel streaks across the width of the plate. This method is non-quantitative, and therefore prevents results by other antimicrobial finishes to be reliably compared. An arguably better method is the agar-diffusion method used in BS EN ISO 20645:2004 which allows a ZOI to be determined due to use of circular fabric samples and total inoculation of the agar surface by spreading. Though this method is semi-quantitative, it does not allow for distinction between inhibition and kill of the microorganism. Quantitative methods such as AATCC 100, BS ISO 13629-2:2014 and BS EN ISO 20743:2013 allow quantitative measurement of the fabric samples bactericidal or fungicidal ability and include the quenching (neutralisation) of the antimicrobial agent as a control. The textile samples are saturated with the test inoculum and bacterial or fungal enumerations are recorded at the start of the experiment and after a contact period of 24 hour for bacterial strain and 48-hour contact for fungal spores. In this chapter, cotton and polyester fabric samples were treated with a

1% CS litsea-lemon EO blend emulsion using the soak-pad-dry method; the treated fabric samples were also investigated for mosquito repellent properties.

6.2 Aims and Objectives

The overall aim of this chapter was to assess the antimicrobial efficacy of fabric (polyester and cotton) when treated with a 1% CS litsea and lemon EO blend (1:2 ratio) emulsion against *E. coli, S. aureus, S. epidermidis*, type and their antibiotic resistant and clinical isolates and the dermatophyte *T. rubrum*

Objectives:

- To qualitatively determine the antimicrobial efficacy of polyester and cotton EO-treated fabrics against *E. coli, S. aureus, S. epidermidis* and their antibiotic resistant/clinical isolates and *T. rubrum* using adapted BS EN ISO 20645:2004 standard;
- To quantify the efficacy of polyester and cotton treated with EO emulsion in the reduction of *E. coli, S. aureus, S. epidermidis, T. rubrum* using adapted BS EN ISO 20743:2013 standard;
- To quantify the efficacy of the EO emulsion in reducing *E. coli, S. aureus, S. epidermidis, T. rubrum* over time;
- To assess the durability of treated polyester and cotton fabrics to standard washing and its antimicrobial activity post-wash.

6.3 Results

6.3.1 *Quantification and Distribution of Major EO Components on Polyester and Cotton Fabric Treated with 1% CS Emulsion*

The percentage liquid pick up was calculated for both treated polyester and treated cotton for three different emulsion with varying concentrations of CS (B1= 1%; B2= 0.75%; B3= 0.5%). The results (Figure 6.1) show that cotton has a higher absorbency for the emulsion compared to polyester; the most viscous emulsion B1 (1% CS) achieved an average liquid pick up of 112.89 ± 1.59 % compared to polyester's average wet pick up of 97.45 ± 5.97 when also treated with B1. Very similar liquid pick-up was achieved for cotton fabric treated with B2 and B3 emulsions, with a liquid pick up of 107.38 ± 4.77% and 107.81 ± 0.76% respectively. The liquid pick up for cotton fabric was significantly higher than polyester for treatment B1 ($p \leq 0.05$), B2 ($p \leq 0.05$) and B3 ($p \leq 0.05$). No significant differences in % LPU were found within treatments for both cotton and polyester ($p \geq 0.05$).

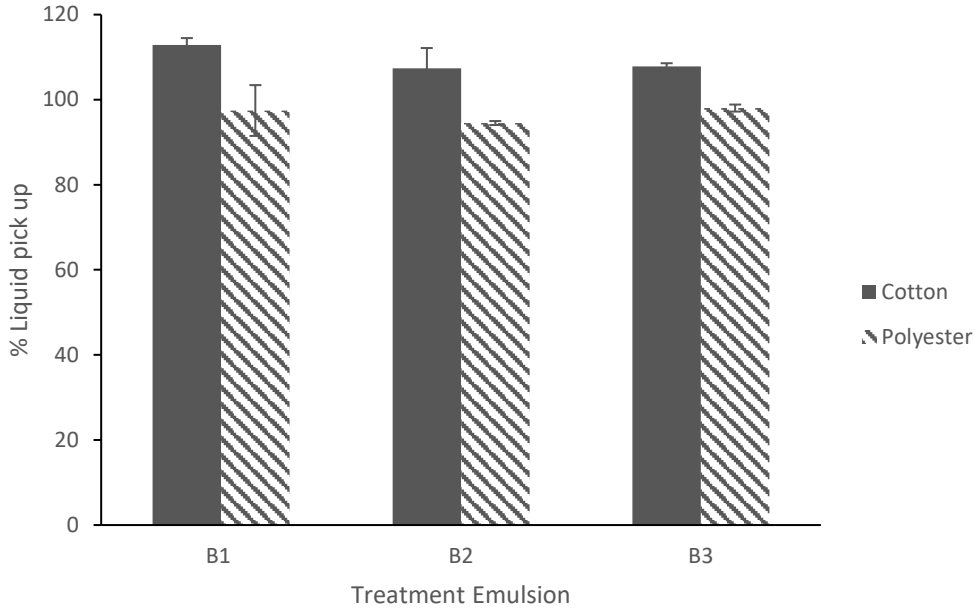
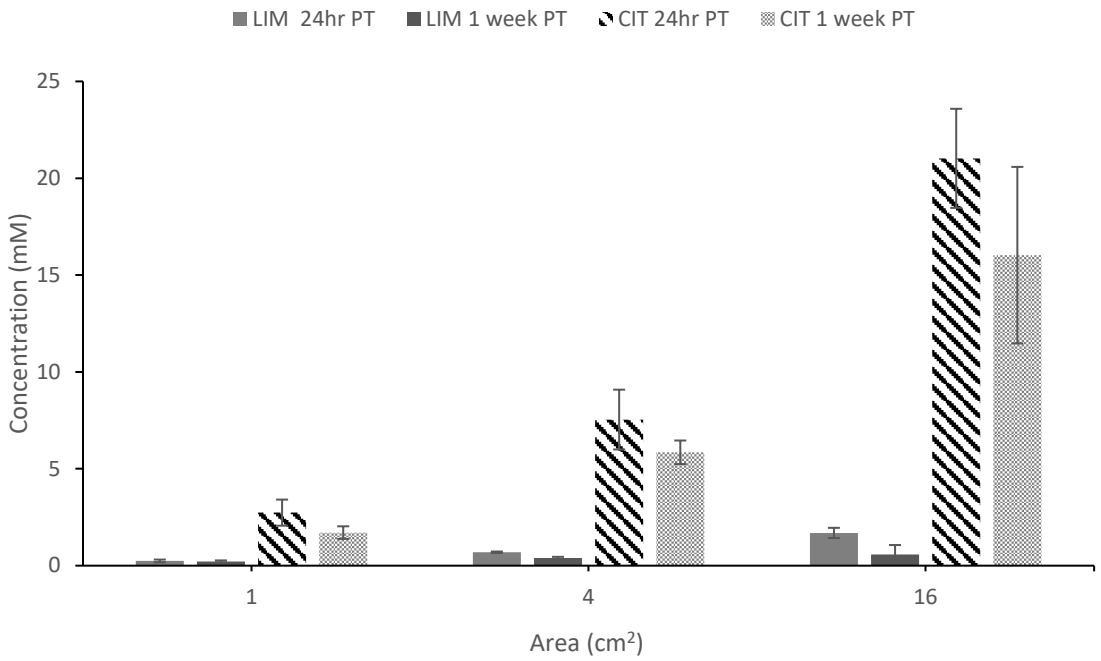


Figure 6.1 Percentage LPU for cotton and polyester fabric treated with emulsions of different chitosan concentration (n=3, ± SD)

Quantification of the main components citral and limonene on treated cotton and polyester fabric was carried out on different areas of fabric samples (1, 4 and 16 cm^2) at 24 h and 1 week post treatment (PT), with significant differences in the concentration of limonene *(*$p \leq 0.05$) on polyester fabric observed (Figure 6.2a) and in the concentration of citral 24 h PT and 1 week PT ($p \leq 0.05$)**.** Differences in the concentration of citral and extracted from cotton (Figure 6.2b) 24 h PT compared to 1 week PT were also significantly different for fabric samples of area 16 cm^2 for limonene and citral ($p \leq 0.05$).

a)



b)

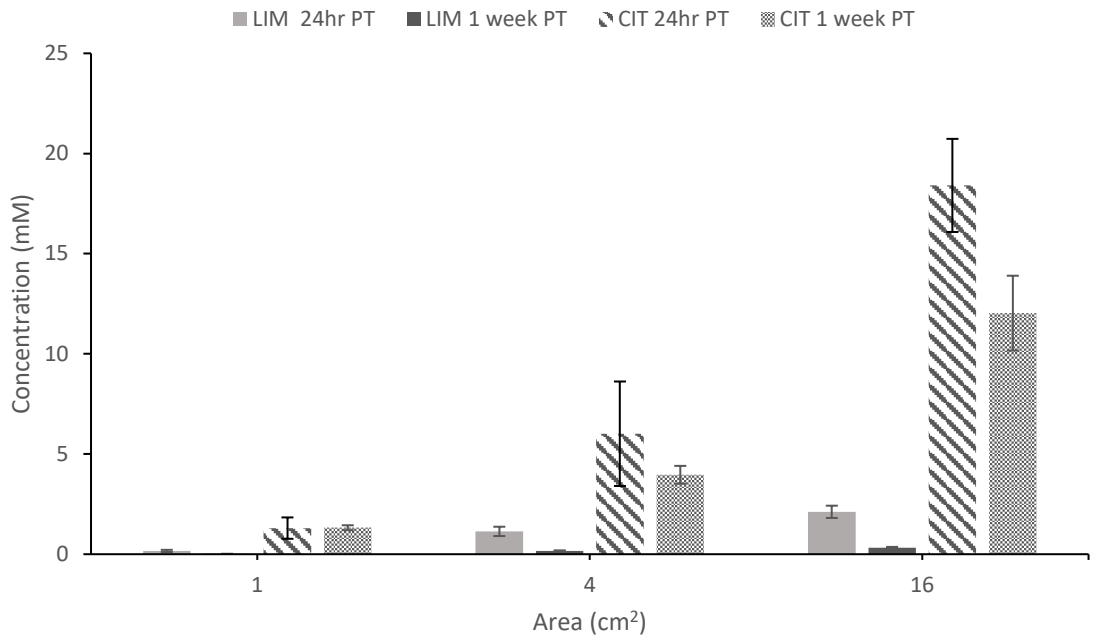


Figure 6.2 Mean concentration (mM) of limonene (LIM) and citral (CIT) compounds extracted from a) treated polyester and b) treated cotton samples of different areas 24 h and 1 week post-treatment (PT) (n=3, ± SD)

6.3.2 *Toxicity Test and Dilution Neutralisation Validation for Treated Fabric*

Neutraliser efficacy test (Figure 6.3) results show that the neutraliser was able to quench the activity of the antimicrobial emulsion, with no significant difference ($p \geq 0.05$) observed between mean $\log_{10}$ CFU/ml of neutralised emulsion compared to the control for *E. coli*, *S. aureus, S. epidermidis* and *T. rubrum*. The efficiency of the neutraliser was also tested using the DNV test for both polyester and cotton treated fabric. *E. coli*, *S. aureus*, *S. epidermidis* and *T. rubrum* growth was not affected by the testing conditions and both treated cotton and polyester fabric samples were successfully neutralised (Figure 6.4) with no statistical significance ($p \geq 0.05$) found between mean $\log_{10}$ CFU/ml of control compared to treated fabric samples subjected to neutraliser for *E. coli*, *S. aureus*, *S. epidermidis* and *T. rubrum* which differed by >1 $\log_{(10)}$ for all organisms.

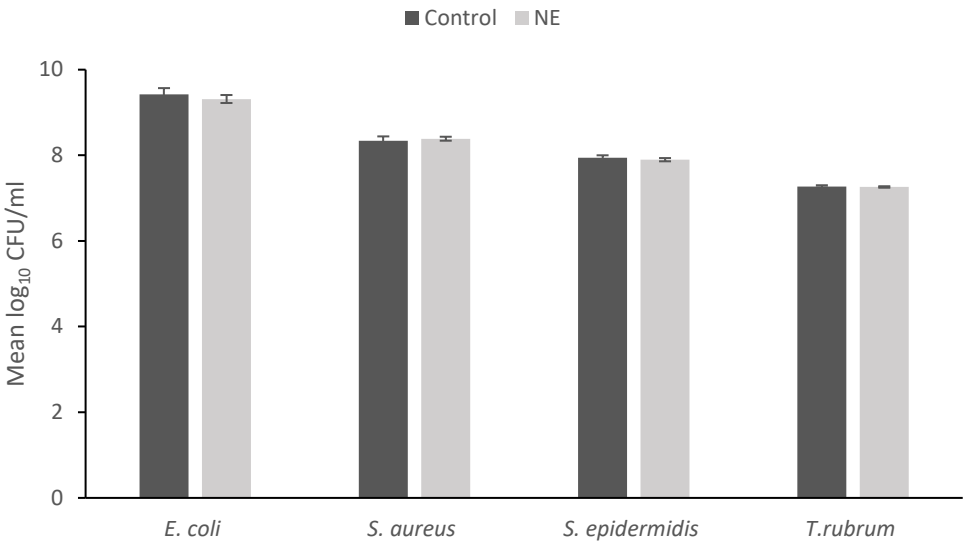


Figure 6.3 Mean $\log_{10}$ CFU/ml of *E. coli, S. aureus, S. epidermidis* and *T. rubrum* after neutraliser efficacy test (NE) compared to control (n=4, ± SD)

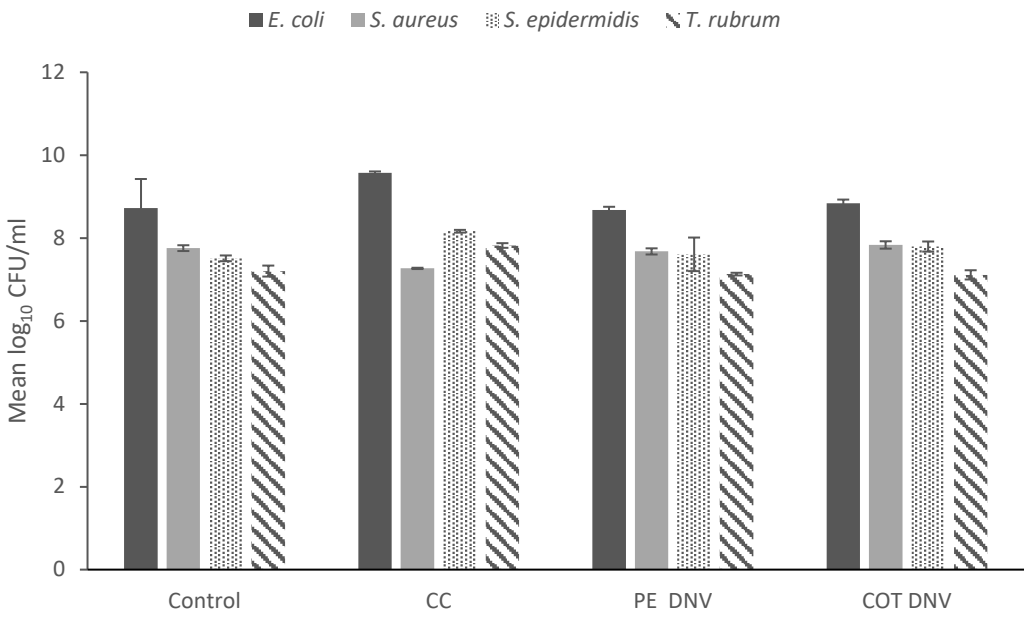


Figure 6.4 Mean $\log_{10}$ CFU/ml of *E. coli, S. aureus, S. epidermidis* and *T. rubrum* under conditions control test (CC), polyester (PE DNV) and cotton (COT DNV) dilution neutralisation test compared to control (n=4, ± SD)

6.3.3 *Qualitative Determination of Antibacterial and Antifungal Activity of Emulsion Treated Polyester and Cotton Fabric post 40°C Wash*

Zones of inhibition were recorded to assess antibacterial and antifungal ability of emulsion treated fabric samples and results (Table 6.1) showed that cotton fabric treated by all 3 emulsions was able to inhibit the growth of *E. coli*, *S. aureus*, *S. epidermidis*, and *T. rubrum* 24 hour post-treatment, with leaching observed against *E. coli* when fabric was treated with B2 and B3 emulsions with ZOI of 4.58 ± 1.69 mm and 0.81 ± 0.60 mm respectively. Leaching was also observed by cotton treated with B2 emulsion (24 h post-treatment) against *S. epidermidis* with a ZOI of 1.43 ± 0.96 mm. *E. coli* appeared to be the most susceptible to treated fabric, with all polyester treatments inhibiting *E. coli* with

ZOIs of 4.64 ± 1.86 mm for B1 treatment, 2.96 ± 2.20 mm for B2 and 1.62 ± 0.36 mm for B3, after 24 hours. *S. epidermidis* showed weak susceptibility to polyester treated fabric, when tested after 24 h, as growth was observed under fabric treated with B1 and B3. *T. rubrum* was inhibited by both cotton and polyester for all treatments, with no growth underneath the fabric, no leaching was observed (no zone beyond the fabric diameter).

Activity of the fabric was retained and improved significantly after 1 week of storage (Table 6.1) with larger zones of inhibition observed by both cotton and polyester samples compared to when fabric was tested 24 h post-treatment. The greatest inhibition was recorded by polyester treated fabric with zones of 19.43 ± 5.46 mm against *E. coli*, 22.31 ± 6.97 mm against *S. aureus*, 9.31 ± 0.51 mm against *S. epidermidis* and 65.74 ± 1.05 mm against *T. rubrum*. There was a significant increase in activity ($p \leq 0.05$) by cotton fabric after 1 week against *E. coli* increasing from an average of 4.58 mm to 9.26 mm.

Table 6.1 ZOIs (mm) for treated cotton and polyester 24h and 1 week post treatment against *E. coli, S. aureus, S. epidermidis* and *T. rubrum* (+) = growth underneath fabric (-) = no growth underneath fabric. (n=6, ± SD)

	Growth under fabric				**ZOI (mm)**			
Fabric	*E. coli*	*S. aureus*	*S. epidermidis*	*T. rubrum*	*E. coli*	*S. aureus*	*S. epidermidis*	*T. rubrum*
24 hr Post-treatment								
Cotton								
B1 Treated	-	-	-	-	0	0	0	0
B2 Treated	-	-	-	-	4.58±1.69	0	1.43±0.96	0
B3 Treated	-	-	-	-	0.81±0.60	0	0	0
Control	+	+	+	+	0	0	0	0
Polyester								
B1 Treated	-	-	+	-	4.64±1.86	0	0	0
B2 Treated	-	-	-	-	2.96±2.20	0	0	0
B3 Treated	-	-	+	-	1.62±0.36	2.64±2.29	0	0
Control	+	+	+	+	0	0	0	0
1 week Post-treatment								
Cotton								
B1 Treated	-	-	-	-	9.26 ± 0.30	9.48 ± 4.81	4.22 ± 2.23	64.96 ± 1.28
Control	+	+	+	+	0	0	0	0
Polyester								
B1 Treated	-	-	-	-	19.43 ± 5.46	22.31 ± 6.97	9.31 ± 0.51	65.74 ± 1.05
Control	+	+	+	+	0	0	0	0

The activity of cotton and polyester fabric treated with 1% CS EO emulsion was screened against MRSA strain and clinical isolates of *E. coli* and *S. epidermidis* at 24 h and 1-week post treatment (Table 6.2), antimicrobial activity was again preserved after 1 week but with a reduction in the zones of inhibition observed against MRSA with polyester samples giving zones of 17.64 ± 1.93 mm after 24h and 1.21 ± 0.71mm, 1 week post-treatment. *S. epidermidis* was also inhibited by both cotton and polyester treated fabric at both time points with the greatest zone observed after 24 h for both cotton (13.29 ± 8.25 mm) and polyester (15.03 ± 6.70 mm) samples.

The assessment of activity of treated cotton and polyester fabric after a standard 40°C wash showed that both fabrics were unable maintain their activity against all bacteria after post-wash. Activity was maintained for treated cotton against *T. rubrum* which resulted in a mean ZOI of 0.52 ± 0.24 mm.

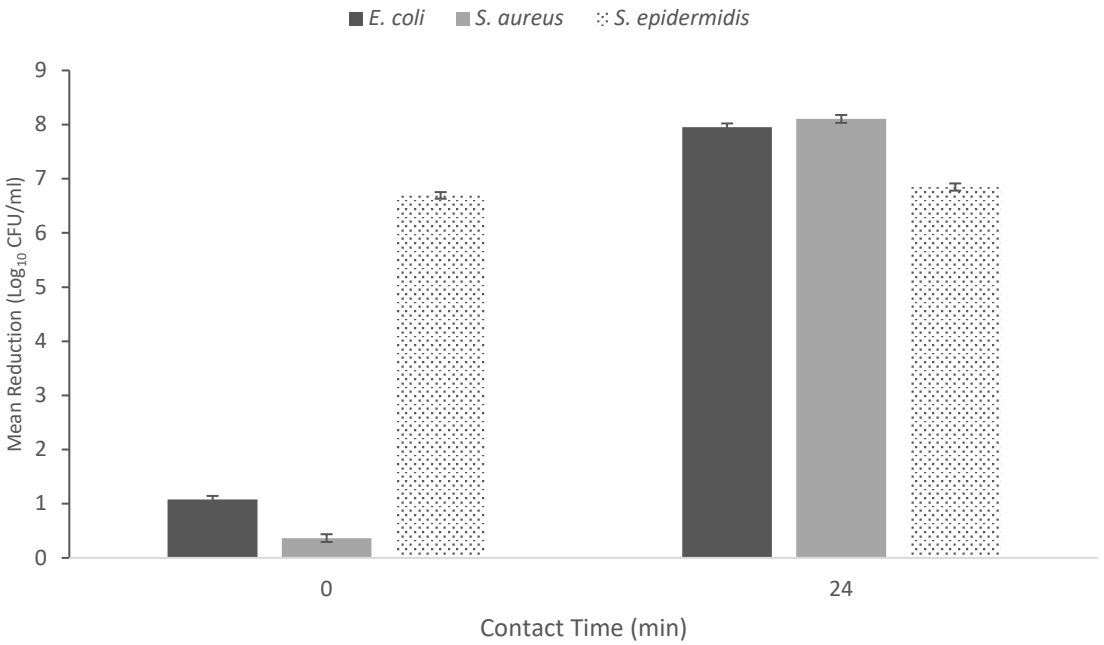
Table 6.2 ZOIs (mm) for *E. coli* (clinical strain 390685Q), MRSA (antibiotic strain NCTC 12497) and *S. epidermidis* (clinical strain PLO 21862) by treated cotton and polyester 24h and 1 week-post treatment. (+)= growth underneath fabric, (-) = no growth underneath fabric. (n=6, ± SD)

	Growth under fabric			Zone of inhibition (mm)		
Fabric type	*E. coli* (390685Q)	*MRSA* (NCTC 12497)	*S. epidermidis* (PLO 21862)	*E. coli* (390685Q)	*MRSA* (NCTC 12497)	*S. epidermidis* (PLO 21862)
24 hr Post-treatment						
Cotton						
B1 Treated	-	-	-	0	7.65 ± 2.22	13.29 ± 8.25
Control	+	+	+	0	0	0
Polyester						
B1 Treated	+	-	-	0	17.64 ± 1.93	15.03 ± 6.70
Control	+	+	+	0	0	0
1 week Post - treatment						
Cotton						
B1 Treated	-	-	-	0.87 ± 0.71	2.44 ± 0.48	1.18 ± 0.32
Control	+	+	+	0	0	0
Polyester						
B1 Treated	-	-	-	0.53 ± 0.47	1.21 ± 0.71	1.10 ± 0.44
Control	+	+	+	0	0	0

6.3.4 *Quantitative Determination of Antibacterial and Antifungal Activity of 1% CS Emulsion-Treated Polyester and Cotton*

Quantitative determination of the antibacterial and antifungal activity of the treated fabric is essential as the screening (qualitative) of the fabric by disc diffusion only gives an indication of antimicrobial activity. The ability for the fabric to reduce bacterial and fungal load was demonstrated using the challenge test (Figure 6.5), the greatest inhibition was observed for *S. epidermidis* with a mean reduction of 6.69 $\log_{10}$ CFU/ml by polyester at 0 min and 6.56 $\log_{10}$ CFU/ml by cotton fabric at 0 min. *S. aureus* growth was reduced by only 0.37 $\log_{10}$ CFU/ml by polyester at 0 min but was able to reduce by 8.11 $\log_{10}$ CFU/ml after 24h, whilst cotton was able to reduce *S. aureus* growth by 6.96 $\log_{10}$ CFU/ml after 0 min CT. The greatest reduction was observed for *E. coli* (Figure 6.5) and *T. rubrum* (Figure 6.6) by treated polyester and cotton was seen after CTs of 24h and 48h respectively, with highest mean reduction observed for *E. coli* of 8.91 $\log_{10}$ CFU/ml by cotton; *T. rubrum* growth was reduced by 6.76 $\log_{10}$ CFU/ml and 6.79 $\log_{10}$ CFU/ml by cotton and polyester respectively after 48 h. Difference in mean reductions between cotton and polyester were found to be statistically significant, with a significant reduction observed by polyester and cotton for *S. aureus* and *E. coli* at 24 h and by *T. rubrum* at 48h ($p \leq 0.05$).

a)



b)

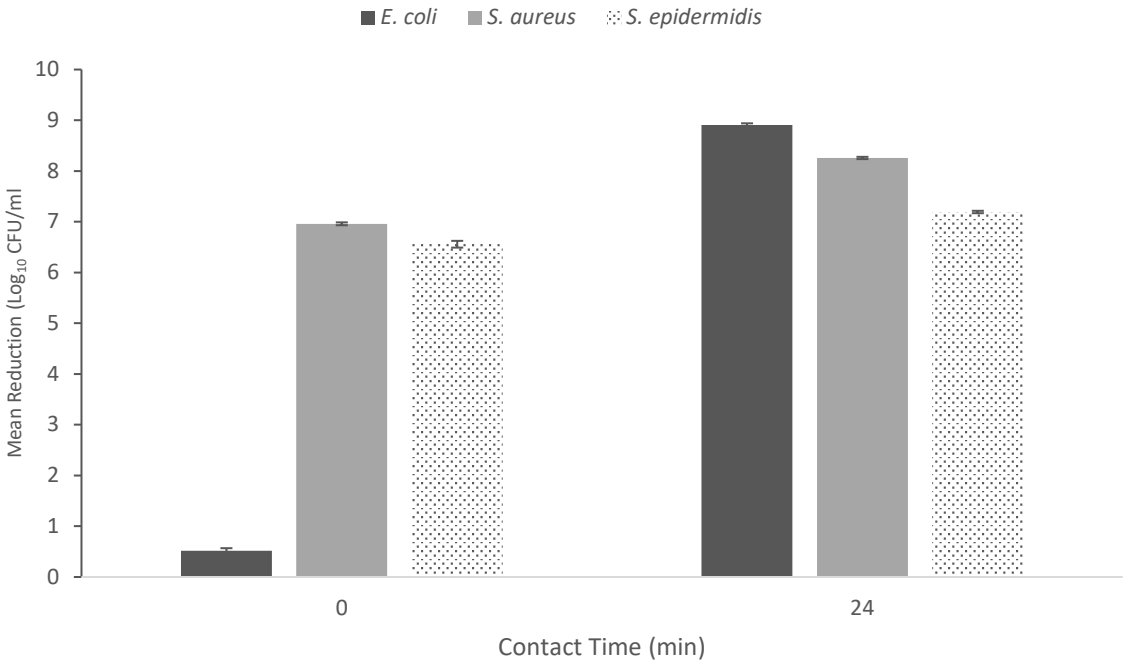


Figure 6.5 Mean reduction from an initial inoculum of 8 $\log_{10}$ CFU/ml of *E. coli, S. aureus* and *S. epidermidis* in the presence of a) polyester and b) cotton (n=4, ± SD)

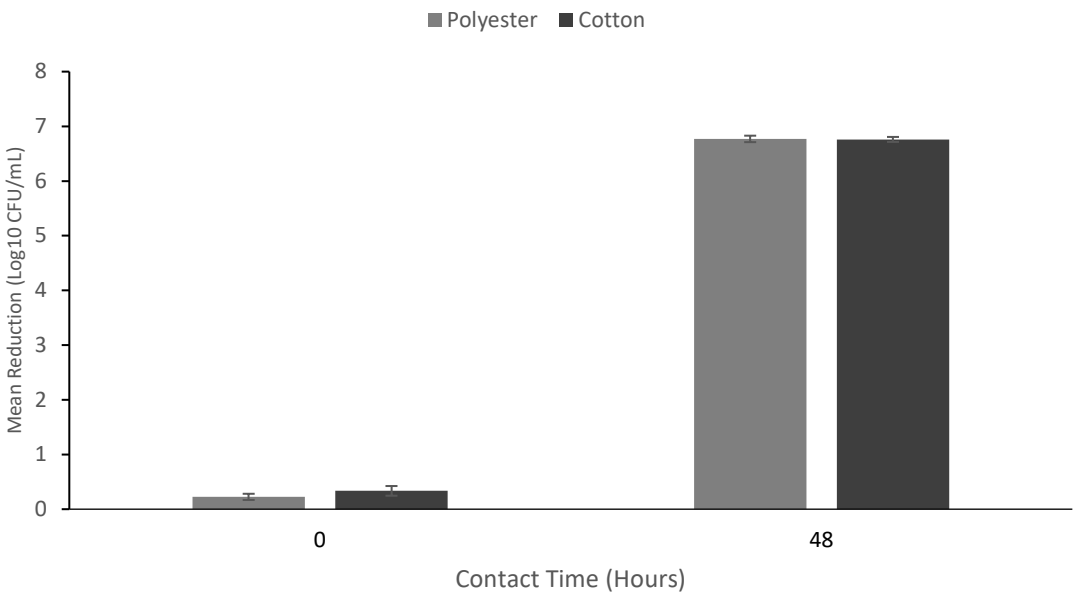


Figure 6.6 Mean reduction from an initial inoculum of 7 log_{10} CFU/ml of *T. rubrum* in the presence of polyester and cotton (n=4, ± SD)

6.3.5 *Antibacterial and Antifungal Activity of a 1% CS EO-Emulsion (Time-Kill Assay)*

Time-kill studies were performed on *E. coli*, *S. aureus*, *S. epidermidis* and *T. rubrum* using emulsion B1 (1% CS) diluted to 1% v/v. *S. epidermidis* bacterial load was completely reduced after a CT of < 2 min, with a reduction of 7.31 log_{10} CFU/ml observed (Figure 6.7); *S. aureus* was reduced by 3.36 log_{10} CFU/ml after 0 min and by 7.54 log_{10} CFU/ml after 5 min of CT. The emulsion did not reduce *T. rubrum* as rapidly (Figure 6.6) when compared with results from the bacterial strains (Figure 6.7) with complete reduction (7.13 log_{10} CFU/ml) observed after 2 hours (120 min) of CT with the emulsion.

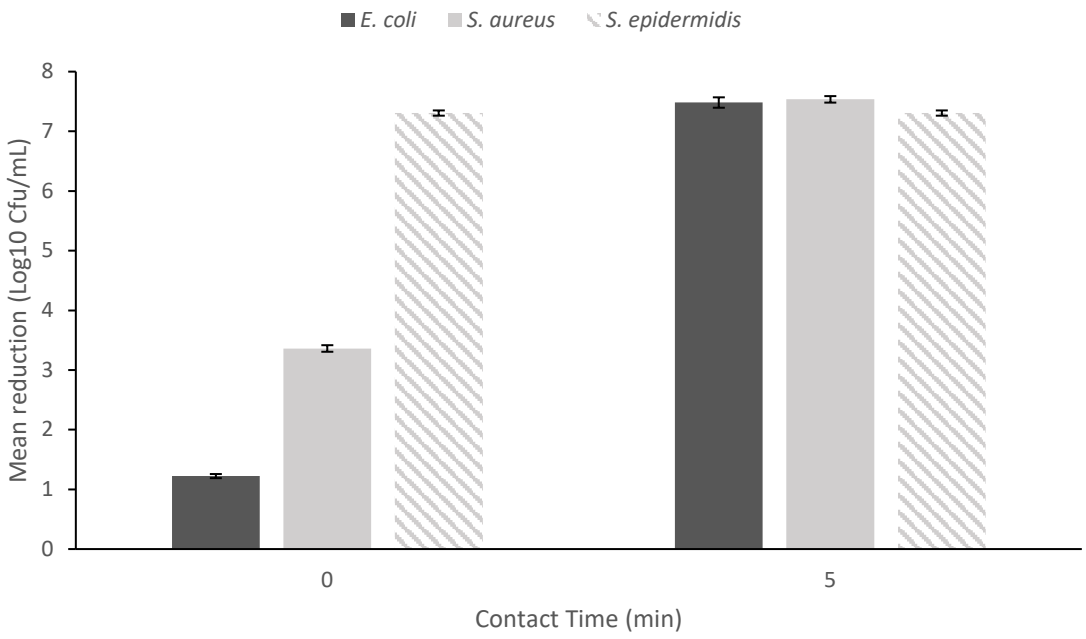


Figure 6.7 Mean reduction from initial inoculum of 7 log_{10} CFU/ml of *E. coli, S. aureus* and *S. epidermidis* at CTs 0 min and 5 min with 1% CS EO emulsion (n=4, ± SD)

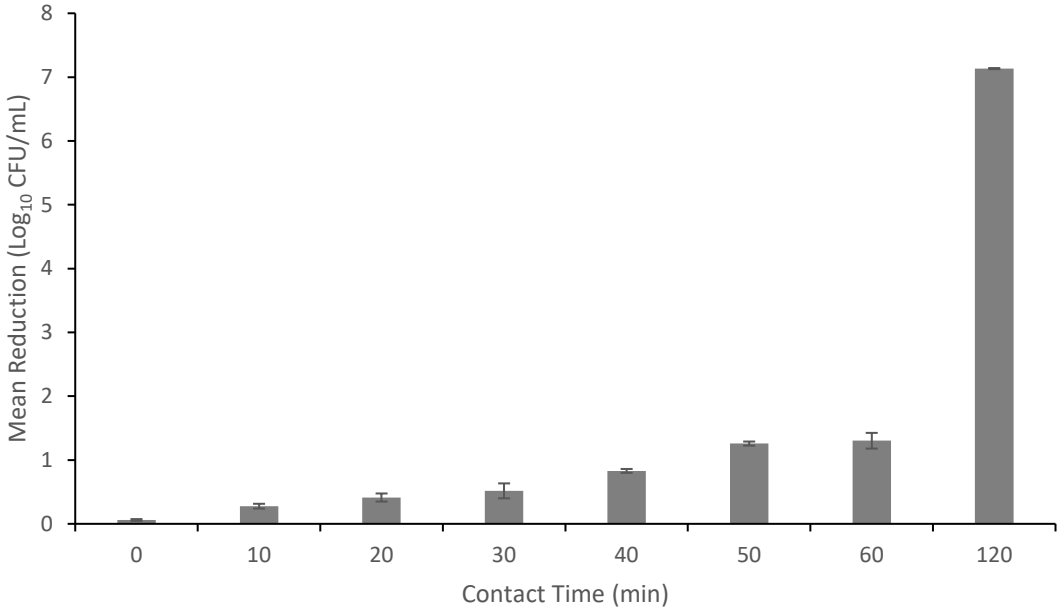


Figure 6.8 Mean reduction from initial spore inoculum of 7 log_{10} CFU/ml of *T*. *rubrum* at various CTs with 1% CS EO emulsion (n=4, ± SD)

6.3.6 *Evaluation of Repellency of Litsea-Lemon EO Blend and Emulsion Treated Cotton Fabric against Aedes aegypti Mosquitos*

Results from the mosquito repellency study showed that when cotton samples were simply impregnated with the litsea-lemon EO blend (1:2 ratio), average recorded repellency against *A. aegypti* mosquitos was 52.94%. When cotton samples were treated with 1% CS litsea-lemon EO emulsion, repellency increased to 71.43% against *A. aegypti,* which was a 18.49% increase in repellency.

6.4 Discussion

The overall aim of this chapter was to investigate the antimicrobial activity of a lemon and litsea EO-emulsion as an antimicrobial finish for natural (100% cotton) and synthetic (100% polyester) textiles against microorganisms related to skin infection. Results from Figure 6.1 demonstrate that both polyester and cotton were able to achieve high levels of percentage liquid pick up (up to 112.89%) of the antimicrobial emulsion by controlling paddling pressure, with cotton samples having significantly higher absorbency for the emulsion compared to polyester samples ($p \leq 0.05$). Although the emulsions (B1, B2 and B3) used to treat the fabric samples differed in viscosity due to the concentration of chitosan used for microencapsulation of EOs, there was no significant difference in the percentage pick up observed by both cotton and polyester between the three treatments, with all three emulsions allowing for high %LPU of 94.5-112.9% (Figure 6.1). The absorbency of the fabric to a finishing treatment is greatly dependent on the type of fibres and the state of the textile before the treatment, which is why scouring is used as a textile cleaning process. Scouring is an important pre-treatment process, especially when dealing with natural fibres, which are more prone to the presence of natural impurities such as oils, waxes, fats, pectic acids and minerals that can contribute in giving the fabric hydrophobic qualities (Marechal et al., 2012) . Loom state cotton, for example, will contain up to 12% of natural impurities of total weight of the fibre, including wax; when waxes on fabric is not removed it leads to a non-uniform absorption of finishes during wet-processing; therefore,

the water absorbency of a fabric needs to be improved by scouring process before finishing (Choudhury, 2006; Karmakar, 1999).

Screening of the finished fabric samples showed that both treated cotton and treated polyester displayed antimicrobial activity against all test microorganism with bacteriostatic activity displayed against *E. coli*, *S. aureus*, *S. epidermidis* and fungistatic activity against *T. rubrum* (Table 6.1). The only exception found with B1 emulsion treated polyester against *S. epidermidis* which was unable to inhibit the growth of *S. epidermidis* (growth observed underneath fabric coupon). Greater inhibition was observed for MRSA (antibiotic resistant strain NCTC 12497) and *S. epidermidis* (clinical isolate PLO 21862) with ZOIs between 7.65 and 17.64 mm observed for MRSA and ZOI 13.29 -15.03 mm for *S. epidermidis* (Table 6.2). No leaching was recorded (no ZOI) for *E. coli* (clinical strain 390685Q) for both cotton and polyester though bacteriostatic activity was still observed directly underneath the fabric samples. A recurring pattern observed in the results is the increase of leaching ability of the encapsulated EOs from the treated textiles, which is represented by the increased ZOIs after 1 week of storage compared to those recorded from testing of 1 day old fabric (24h) as seen in Table 6.1 and Table 6.2. This suggests that there is a change occurring within the treated fabric with storage that allows for greater release of encapsulated EOs from the textiles; after 1 week of storage, polyester and cotton fabric showed an increased ZOI from 0 mm for both, to 64.96 ± 1.28 mm and 65.74 ± 1.05 mm respectively, against *T. rubrum.* Due to storage of the fabric at room temperature it is possible that changes in the composition of the

encapsulated EO may have occurred over time; Mehdizadeh et al (2017) reported higher increase in compounds of cumin EO with lower boiling points when stored at room temperature (Mehdizadeh et al., 2017). It is therefore possible that the same may have occurred with the EO blend within the emulsion during the storage period, therefore increasing the activity. Untreated (control) fabric samples clearly displayed no antimicrobial activity, failing to inhibit the growth of antibiotic resistant, clinical and type strains of bacteria and the dermatophyte *T. rubrum* within the area in contact with the textile sample. A study by Walentowska et al (2013) on the antimicrobial activity of linen-cotton blended fabric (55% linen and 45% cotton) found that the growth of the moulds *A. niger*, *Chaetomium globosum, Gliocladium virens, Paecilomyces variotii* and *Penicillium ochrochloron* was completely inhibited when the fabric blend was treated with 8% thyme EO in methanol, and by comparison control samples (untreated fabric) were completely covered by moulds and the fabric was decomposed (Walentowska and Foksowicz-Flaczyk, 2013).

The three emulsions used to treat the fabric in the preliminary screening, differed in the concentration of CS used to formulate the emulsion, however, the emulsion presented similar ability to inhibit the test microorganisms, except with PE against *E. coli* where ZOI decreased as the concentration of CS decreased giving ZOI for B1 (1% CS), B2 (0.75% CS) and B3 (0.5% CS) of 4.64 ± 1.86 cm, 2.96 ± 2.20 cm and 1.62 ± 0.36 cm respectively (Table 6.1). The emulsion with the highest concentration of CS (1%) was therefore used for further treatments and investigations. Incongruity of some of the results may be attributed to the

agar diffusion method, although it is often used for assessing bacteriostatic and fungistatic activity of finished textiles; weaknesses in the method can be highlighted as it does not allow for quantitative analysis as it cannot differentiate cell death and inhibition of growth and makes it more difficult to make comparisons with other types of fabric finishes.

Many EOs are antimicrobial but do not gain attention due to poor antimicrobial longevity caused by their volatile nature (Javid et al., 2014). The microencapsulation of natural compounds is used to improve the durability on fabrics of natural agents like EOs and the process involves encapsulating the EOs by using a polymeric wall material such as chitosan, sodium alginate or combinations of polymers; the resulting finish can then be applied onto textile materials using different methods. The encapsulation of litsea and lemon EO showed promising results; when diluted to 1% v/v the emulsion was able to completely reduce the bacterial load by 7-log after a zero CT for *S. epidermidis* and within 5 minutes of CT for *E. coli and S. aureus* (Figure 6.7). In comparison the kill time was slower for *T. rubrum* and the emulsion was able to completely inactivate the dermatophyte spores after 120 minutes of CT (Figure 6.8). Tian et al (2016) carried out a time-kill investigation on a 10% cinnamaldehyde nanoemulsion and reported that it was only able to reduce the bacterial load of *E. coli* by less than 1-log after 4 hours of CT, and between 4-9 hours bacterial load returned to nearly initial levels (Tian et al., 2016). A 0.25% lemon-myrtle oil emulsion formulated by Buranasuksombat et al (2011), however, was able to achieve a 2-log reduction in bacterial count of *E. coli* and *Bacillus cereus* after a

zero-CT, these increased to a total reduction (around 8-log) after 15 min of CT (Buranasuksombat et al., 2011).

Textile challenge test results show that treated polyester and cotton fabric possessed good antimicrobial activity, with complete reduction of bacterial cells achieved by both treated fabrics after 24 h CT for all test bacterial organisms - *E. coli*, *S. aureus* and *S. epidermidis* (Figure 6.7). In line with results seen with the nanoemulsion-time kill, *S. epidermidis* was most susceptible to treated fabrics, achieving over 6-log reduction after zero CT. *T. rubrum* spores were completely inactivated by treated fabrics after 48 h CT, with about 7-log reduction achieved (Figure 6.6). Time-kill bioassays for EO treated textiles have not been widely reported within the literature, though a study on 100% woven cotton treated with geranium EO reported a reduction of *S. aureus* of 100% for cotton treated with pure geranium extract, a reduction of 92% and 94% for cotton treated with microencapsulated geranium EO by coacervation-spray drying and spray drying respectively (Thilagavathi and Kannaian, 2010). Similarly, a study by Khodary et al (2017) treated cotton wound dressing with microencapsulated geranium leaves extract using the pad-dry-cure method observed a 100% reduction after a 4-hour CT for both *E. coli* and *S. aureus* (Khodary et al., 2017). *Aloe vera* is another eco-friendly and naturally occurring antibacterial agent that has been investigated for use as textile finish; Ali et al (2014) conducted a study on *Aloe vera* gel (3% w/v) finished cotton fabric and observed over 90% reduction of *E. coli* and *S. aureus*, this increased to 99% with increasing concentration (up to 7%) of *Aloe vera* (Ali et al., 2014). Quantitative antimicrobial tests have also been carried out on cotton

fabric coated with plant extracts of pomegranate, neem and turmeric, with *E. coli* reduction at 3% concentration of 62.83%, 30.66% and 37.48% respectively, all of which increase with a 5% concentration to reductions of 82.42%, 39.77% and 46.65% respectively (Mahesh et al., 2011).

More studies are required on natural fabric finishes activity against dermatophytes like *T. rubrum*; it was reported by Ovidiu et al (2016) that a cotton (56%) and polyester (44%) blend treated with rosemary EO and orange EO achieved a maximum reduction rate of for dermatophyte *Epidermophyton floccosom* of 56.99% by the rosemary EO treatment and 92.48% by orange EO treatment (Ovidiu et al., 2016). Results achieved in this study using the control (untreated fabric) samples (Figure 6.4) showed that the control fabric did not possess any antimicrobial properties, which is important when assessing whether the activity observed is solely attributable to the finishing treatment; these results complement those from the qualitative screening of the treated fabric (Table 6.1) where growth of all organisms was not visually inhibited by the untreated fabric coupons. A study by Ghayempour and Mortazavi (2015) on cotton fabric treated with peppermint oil loaded nanocapsules also reported a 100% bacterial reduction for both *S. aureus* and *E. coli*, however the untreated fabric appeared to possess antibacterial activity with a reduction of 66% and 65% observed for *S. aureus* and *E. coli* respectively (Ghayempour and Mortazavi, 2015).

The wash tests suggest that currently the microencapsulated EOs would only be suitable for single use; this is not surprising because the emulsion is water

soluble. Single use antimicrobial textiles could be utilised for wound care, however further studies on toxicity would need to be carried out to determine the human sensitivity to the EO blend when used topically. Reports of sensitivity have also been published for peppermint EO by Nair (2001) and it has been argued that this sensitivity could be attributed to a lack of selectivity of EO compared to antibiotics which have selective toxicity against pathogens, thus sparing the hosts' cells (Nair, 2001; Owen and Laird, 2018). A clinical trial by Aspres and Freeman (2004) which used tea tree EO determined that at a concentration of 5% the pure EO could cause irritation, however, a different study by Veien et al (2004) did not record any irritation when a patch test was carried out with 10% tea tree EO (Aspres and Freeman, 2004; Veien et al., 2004). Orchard and van Vuuren (2017) also argue that patch tests are not fairly indicative of real-world use of an EO containing product as there are reports of only mild sensitivity for tea tree EO (Caelli et al., 2000b; Dryden et al., 2004; Enshaieh et al., 2007; Orchard and van Vuuren, 2017). Due to potential variation in toxicity between batches of EOs, published toxicity results on EOs cannot be considered individually and therefore standardised methods for EOs need to be developed for successful application of novel EO based formulations (Orchard and van Vuuren, 2017; Owen and Laird, 2018).

Nevertheless, EO also show promise within other fields, as shown by the mosquito repellency results with 71.43% repellency observed when cotton samples treated with 1% CS emulsion were tested. When a neat blend of 1:2 litsea-lemon EO blend was used to impregnate cotton samples and tested for

repellency however, the repellency effect was reduced to 52.94%, indicating the importance of encapsulating EOs, as the results can be linked to the high volatility of EO components such as limonene and citral; limonene itself has been previously explored for insecticidal activity with cotton samples (Hebeish et al., 2008). Interestingly, a study by Trongtokit et al (2005) on the repellency of EOs to mosquito bites to *A. aegypti* reported 0% repellency by undiluted litsea EO, and at concentrations of 10% and 50%, though the method used in this study was the arm-in-cage test (Trongtokit et al., 2005).

The new regulations by the EU Directive 98/8/EC, have now enforced the elimination and withdrawal from the market of many commonly used biocides and products which are based on substances which are exceptionally toxic to humans and the environment. Research into eco-friendly antimicrobial agents for textiles has therefore been on the increase for use in the finishing process of textiles, such as medical and health-care textiles, as the use of plants in the creation of biocides can results in low-cost, eco-friendly and very effective biocides and antimicrobial fabrics. The litsea and lemon EOs encapsulated in a CS and SA emulsion in this study showed good antimicrobial activity during *in-vitro* time kill assays with a 7 $\log_{(10)}$ reduction at time 0 against *E. coli* and after a contact time of 5 min against *S. aureus*. A 100% reduction was also achieved for *T. rubrum* after 120 min of contact time. This activity was retained upon application of the emulsion on synthetic and natural textiles, with the best results seen for treated cotton showing a >6-fold reduction of *S. aureus and E. coli* ($p \leq 0.05$), indicating that EOs show promise in their use as finishing for the creation of eco-friendly

functional antimicrobial textiles and potential in the development of mosquito-repellent textiles.

Chapter 7. Discussion

EOs have many actual uses in food preservation, pharmaceutics, natural therapies and cosmetics. The antimicrobial and antifungal properties of ten EOs were compared against two Gram-positive bacterial species (*S. aureus* and *S. epidermidis*), two Gram-negatives (*E. coli* and *P. aeruginosa*) and a dermatophyte (*T. rubrum*) associated with skin conditions, with an aim to develop an effective EO-based emulsion. *S. aureus* and most specifically Methicillin-resistant strains can be found to colonise the skin and wounds of 63-90% of patients and have been carefully monitored in hospitals (Caelli et al., 2000a; CDC, 2014; Gallagher et al., 2017). *S. aureus* has developed resistance against erythromycin, quinolones, mupirocin, tetracycline and vancomycin (Boucher et al., 2009); many EO investigations have focused on one specific EO (*M. alternifolia*) against *S. aureus*, and though they have shown great promise, other EOs like *C. limon* and *L. cubeba* used in this study, and their blends, have mostly been neglected MRSE has become a problematic microorganisms due to multidrug resistance; chamomile and oregano are among the EOs that have shown good *in vitro* activity against *S. epidermidis* (Opalchenova and Obreshkova, 2003; Orchard and van Vuuren, 2017). Gram-negative bacteria also present a serious threat with regards to antibiotic resistance and especially *P. aeruginosa* and *E. coli* (Boucher *et al,* 2009; CDC, 2013); they are often found to colonise wounds caused by multidrug resistance (Sienkiewicz et al., 2014; Sienkiewicz et al., 2017).

A number of EOs have antimicrobial activity against *E. coli* and *P. aeruginosa* with the majority of studies focusing on *E. coli* (Orchard and van Vuuren, 2017). Gram-negative pathogens appear to be more resistant to EO inhibition compared to Gram-positive bacteria and this has also been demonstrated in this present study the inhibition of *P. aeruginosa* required higher MICs for lemon, litsea and rosemary EOs (>40 µl/ml, 10 µl/ml and 20 µl/ml respectively) compared to *S. aureus* and *S. epidermidis* which, for example, gave lowest MICs of 1.25 µl/ml and 0.6 µl/ml for litsea (Table 4.2). The low sensitivity of *P. aeruginosa* to EOs has also been observed in studies on various EOs against *P. aeruginosa*, including lemongrass, peppermint, caraway, anise, fennel, geranium, clove, and lavender EOs having MICs >16 µl/ml, which was the maximum concentration tested (Tarek et al., 2014). There is 10-20% risk of a person contacting a dermatophyte infection, including tinea pedis and treatment is costly and onerous due to resistance and side effects (Bajpai et al., 2009). EOs present a good option to treat human fungal infections, though only tea tree EO has been extensively investigated against dermatophyte infections (Orchard and van Vuuren, 2017); in this study *T. rubrum* showed high sensitivity to cotton and PE fabric when treated with a 1% CS encapsulated litsea-lemon EO blend emulsion (Figure 6.6).

The potential of EOs for use in various applications is therefore suggested by their broad spectrum of antimicrobial activity, evident in this investigation as EOs such as litsea, lemon and rosemary showed activity against all organism tested (Table 4.1). When litsea and lemon EO were combined at a 1:2 ratio,

synergism was noted (Table 4.3) against *S. aureus*, *E. coli* and *T. rubrum*, reducing the MICs of the individual EOs; most other combinations gave antagonistic or additive effects. Synergism between EOs is essential when trying to prevent antimicrobial resistance and increasing the antimicrobial activity, however not all combinations will be synergistic. A study on 45 EOs combined with *L. angustifolia* by de Rapper et al (2013), observed that majority of the combinations resulted in indifferent or additive interactions and the few synergistic interactions were mostly against *C. albicans* (de Rapper et al., 2013). A study on the commercial product “Olbas” and the individual EOs in the product were tested separately and in combination and found that there was no further enhancement in the antimicrobial activity when combined (Hamoud et al., 2012).

The litsea and lemon EO blend (1:2 ratio) in this present study was encapsulated with CS and SA (as emulsifiers and stabilizers) within an emulsion. The main challenges faced with the application of natural products like EOs is their durability, shelf-life and antimicrobial efficiency which is why further research must be carried out to evaluate their use in the formation of bioactive textiles (Ali et al., 2014). CS concentration within the emulsions had an effect on the physical stability of the emulsion, with CS concentrations of 0.75% and 1% showing moderate stability over 14 days and a CI of about 10% compared with the lowest CS concentration formulation (0.5%) displaying a CI of over 50% within 5 days of storage (Figure 5.21). Though creaming was observed within 24 hours for all emulsion formulations, this is reversible by simple shaking of the emulsion which disperses the droplets again, which are still surrounded by a protective film and

behave as a single drop. This could be seen by the observation of microscope images of shaken emulsions over 24 hours post- preparation (Figure 5.12) where the droplets observed showed individual droplets surrounded a dark CS-SA film layer indicating the emulsion was easily re-dispersible. This re-dispersibility of the emulsion allowed for the emulsions to be successfully used for treatment of the fabric despite creaming and stability and though differences in viscosity (Figure 5.11) of the three emulsion formulations were observed this was not reflected in the percentage LPU by cotton and polyester (Figure 6.1) as no significant difference was observed in LPU between the three formulations.

Citral and limonene, the main compounds present in litsea and lemon EOs respectively are linked to the antimicrobial activity of the two EOs; when citral and limonene were screened for activity against bacteria, results showed that citral has greater antimicrobial activity against *S. aureus*, *S. epidermidis* and *E. coli* (Figure 5.3). Citral is a mixture of two geometric isomers, neral and geranial and both those isomers are present in both litsea and lemon EOs as identified by GC-MS and shown in Figure 5.4 and Figure 5.5; this may explain the synergistic effect experienced by the blend of the two EOs (Table 4.3). Citral vapour has been reported to have antimicrobial activity against *S. aureus* isolates, with mean ZOI of 5.91 cm and a reduction of 2.03 $\log_{10}$ for MSSA 110 isolate; the study also reported no activity of citral both by disc diffusion and in-vitro against *P. aeruginosa* (Phillips et al., 2012). MICs between 0.03-0.06% v/v have been determined for citral against *E coli* O157, *S. aureus*, *B. cereus*, *Campylobacter jejuni* and *Lysteria monocytogenes* in both vapour and neat form (Fisher and

Philips, 2008). Citral has been recently encapsulated into nanostructured lipid carriers to improve and prolong its efficiency and a study by Mokarizadeh et al (2017) reported lower MIC/MBCs for citral against *S. aureus, B. cereus*, *E. coli* and *C. albicans* when encapsulated (Mokarizadeh et al., 2017). The higher presence of citral was also observed when the presence of citral and limonene on the samples of treated cotton and polyester was quantified (Figure 6.2). When the *in vitro* release of the EO was assessed using citral and limonene as markers, the release profiles were characterised by an initial rapid burst release, followed by a slowed sustained release (Figure 5.19 and Figure 5.20). The same release profile was observed by Esmaelli and Asgari (2015) for *Carum copticum* EO and chitosan nanoparticles and by Kotronia et al (2017) for oregano EO-β-cyclodextrin complexes (Esmaeili and Asgari, 2015; Kotronia et al., 2017).

Citral and limone are both vulnerable to oxidation (Djordjevic et al., 2007) and when the long term chemical stability of the emulsions over 28 days at 15 ºC and 40ºC was assessed citral concentrations after 28 days varied greatly with a nearly 200% increase observed for formulation B1 at 40ºC but a complete decrease (100%) at 15ºC (Figure 5.23); limonene in turn could only be recovered at extremely low concentrations or not at all with fluctuating concentrations observed (results not shown). Turek and Stintzing (2012) reported degradation of EO component (α-terpinene) in rosemary EO when stored at 38°C (Turek and Stintzing, 2012).The storage temperatures used in this study (15°C and 40°C) did not have any significant effect on the chemical stability of the EOs within the emulsion; though higher temperatures than 40°C were not tested during storage,

DSC and TGA results showed that the EOs (individually and their blend), citral, limonene and the emulsions had boiling points far above 40°C and therefore higher stability testing temperature were unlikely to give significantly different results (Figure 5.17 and Figure 5.18).

A different encapsulating method and further stability tests would need therefore need to be carried out e.g. at 4ºC and in an oxygen regulated environment to assess whether these parameters would have a positive effect on the chemical stability of the encapsulated EOs. Due the chemical instability of the EOs, only fresh emulsions were used during the treatment and analysis of cotton and polyester fabric; notably, when changes in the presence of citral and limonene within the treated fabric were evaluated a significant difference was seen in the presence of limonene and citral on treated cotton at 24h PT treatment and their presence on the fabric 1 week PT (Figure 6.2).

The antimicrobial activity of the treated fabric against *E. coli*, *S. aureus, S. epidermidis* and *T. rubrum* test strains and *E. coli* (clinical isolate 390685Q), *S. epidermidis* (clinical isolate PLO 21862) and MRSA (antibiotic resistant strain NCTC 12497) showed an increase after 1 week of storage for type strains (Table 6.1) but a reduction for MRSA and *S. epidermidis* clinical isolate (Table 6.2) for both cotton and polyester. There is strong evidence that this difference in antimicrobial activity retention observed between type and clinical strains in Table 6.1 and Table 6.2 cannot be attributed to homogeneity in the distribution of the emulsion within the fabrics as quantification of citral and limonene within the fabric

demonstrated that there is a proportional decrease in the concentration of the compounds within the fabric as the sample size decreased (Figure 6.2). Concentration of the compounds was proportional to the size of the samples even after 1 week of storage suggesting that there is a direct relationship between the entrapment of the EOs (within the emulsion) on the fabric and the fabric area (Figure 6.2). This in turn suggests that the treatment of the fabric will also be potentially successful at both a small and larger scale, making the method scalable.

When testing for the durability of the fabric to a standard 40°C wash cycle, both polyester and cotton were not able to withstand the washing conditions and the activity of the fabric was not retained (with the exception of cotton against *T. rubrum*); this may indicate that the formulation and fabric treatment method may be more suitable to a single-use product rather than a reusable one. Single-use cotton fabric treated with microencapsulated mixtures of lavender, fennel and laurel EOs has been developed for use in bee-repellency (Eyupoglu et al., 2018).

7.1 Future Studies

Further research is needed, including an investigation on whether the litsea/lemon EO-blend is able to enhance the action of antibiotics against pathogenic wound bacteria such as was explored in a study which found that synergistic effects were found for basil, clary sage and rosemary EOs with antibiotics (Sienkiewicz et al, 2017). To minimize creaming, the droplet size

needs to be reduced to a nano scale (20-200 nm) in order to keep the difference in the density of the two phases (the oil phase and aqueous/polymer phase) as small as possible, by increasing the viscosity of the continuous phase. A method such as ultrasonic emulsification could be explored, in which high-intensity ultrasound waves are applied and can have the advantage of smaller droplet size, lower polydispersity and therefore higher physical stability of the emulsion (Hashtjin and Abbasi, 2015).

The potential for dual-function textiles means further exploration of other possible applications of the treated fabrics also needs to be evaluated, including more robust tests such as the arm-in-cage test for the evaluation of mosquito repellency (Trongtokit et al., 2005). The EOs of litsea and lemon were successfully applied onto cotton and polymer fabric using an eco-friendly method without any toxic fixing agents such as formaldehyde, although when tested for washing durability the activity was not retained apart from the dermatophyte which was highly sensitive to the EO blend. The use of eco-friendly pigment binders (e.g. poly-acrylate) post-treatment could be investigated to see if the durability of the treated fabric is improved post-wash.

This study showed promising results for the use of EOs in the finishing of textiles, although the results from the wash durability test suggests that the fabric treatment antimicrobial activity is temporary and possibly more suited to single use. To solve this problem, the use of a binder would be employed. Research into an eco-friendly binder compatible with the wall materials used in the

encapsulation process would need to be further investigated to improve the staying ability of the encapsulated EOs post-wash. Sensitivity tests on skin would also need to be carried out to assess skin tolerance to the treated fabric.

7.2 Conclusion

In conclusion, this study has shown that the encapsulation process of the EOs tested can be considered as an inexpensive and efficient technique to maintain the antifungal and antibacterial activity of the EOs and enable the application of the EOs onto fabrics. The treatment of fabric has shown to have a potential application in the use of antimicrobial wound dressing or sportswear to aid in the combat of bacterial and fungal pathogens. With the rapid prevalence and emergence of antibiotic resistant clinical and community pathogens, limited number of antimicrobial agents available for the treatment of skin infections by multi drug resistant strains, the use of the litsea/lemon EO blend both as an emulsion and embedded within fabric could be the answer to problems within the clinical arena and sports industry.

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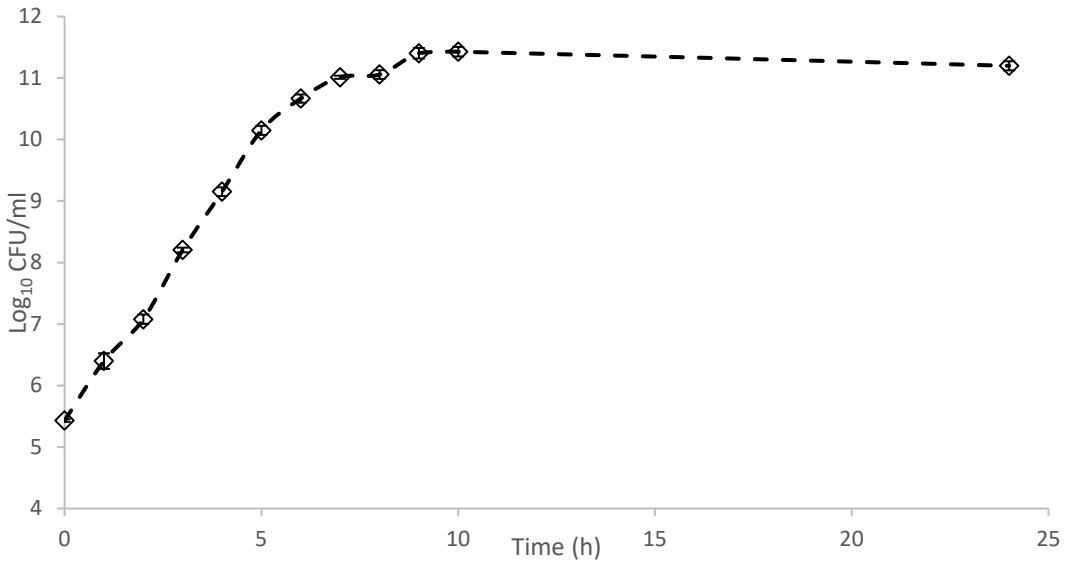
a)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Log10 CFU/ml
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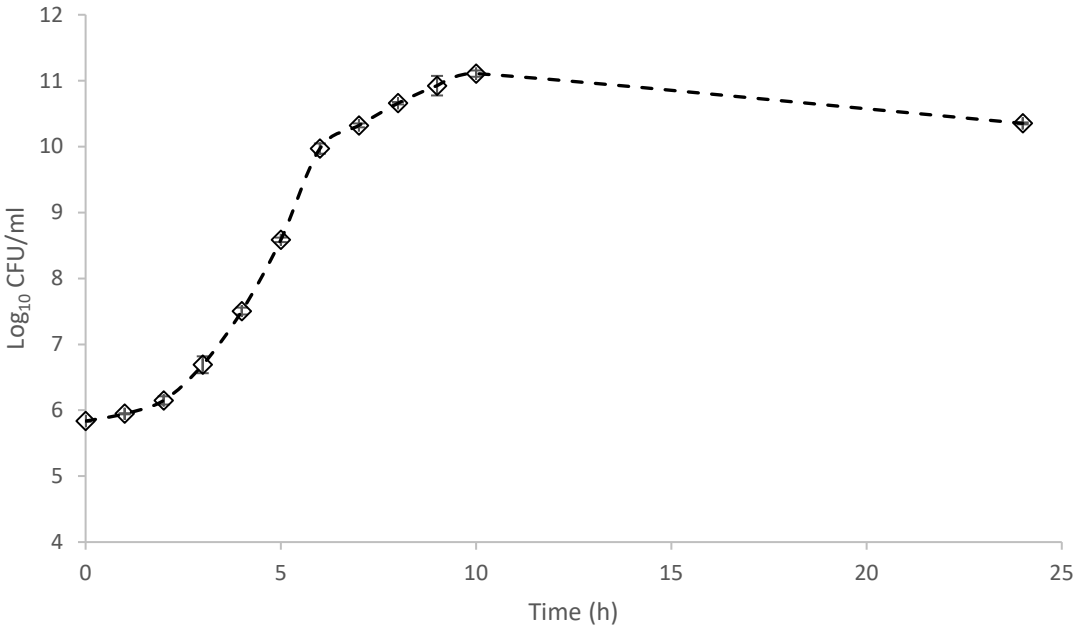
Time (h)

b)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Time (h)

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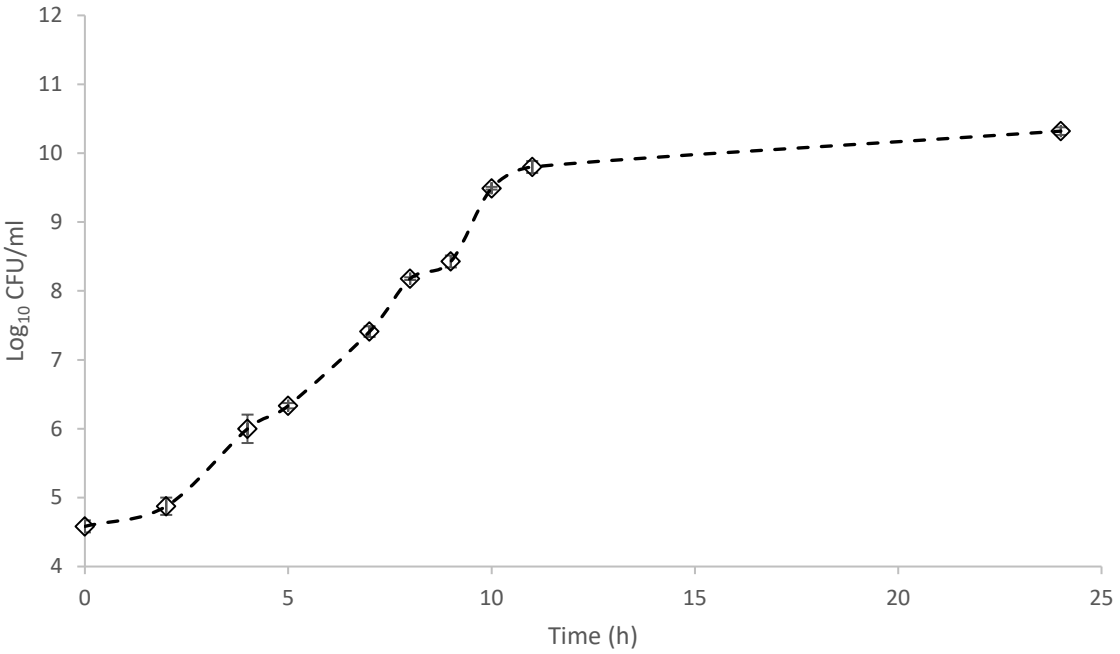


d)

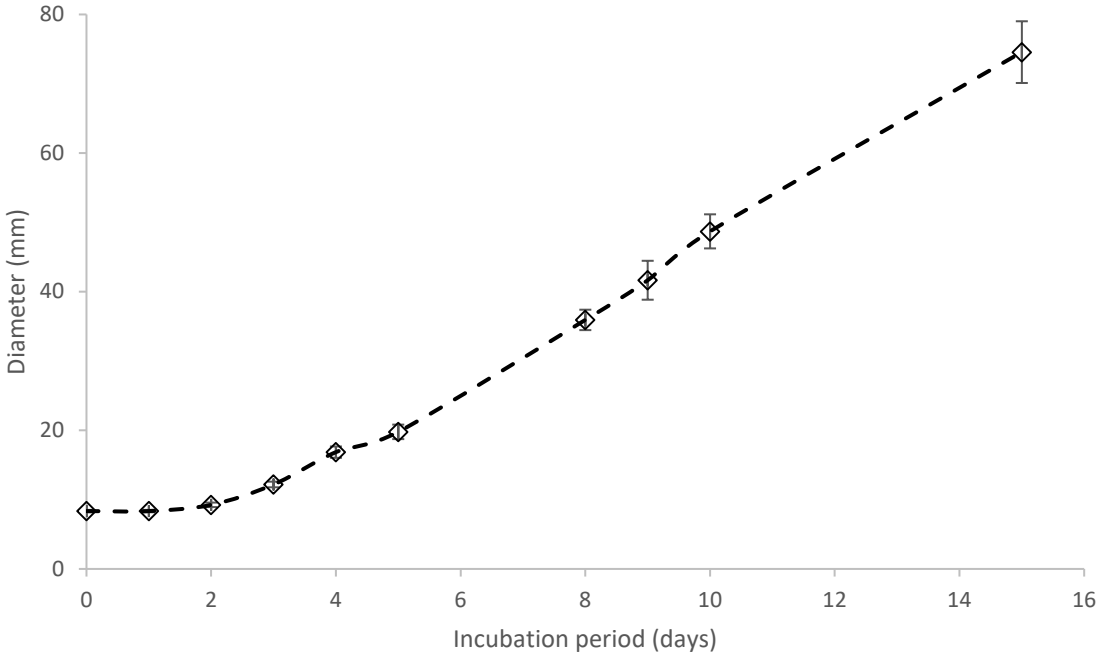


Figure Appendix II: Growth curves of a) *E. coli*, b) *S. aureus*, c) *S. epidermidis* at 37 C and radial growth of d) *T. rubrum* at 30 C.

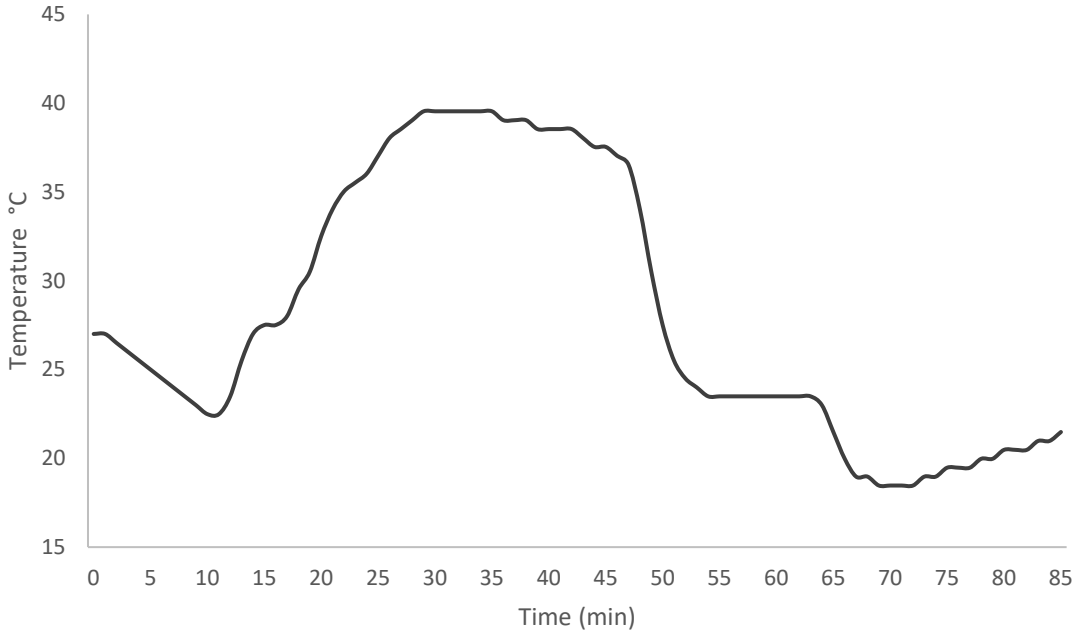


Figure Appendix II: Fabric wash cycle temperature log

Tests of Normality

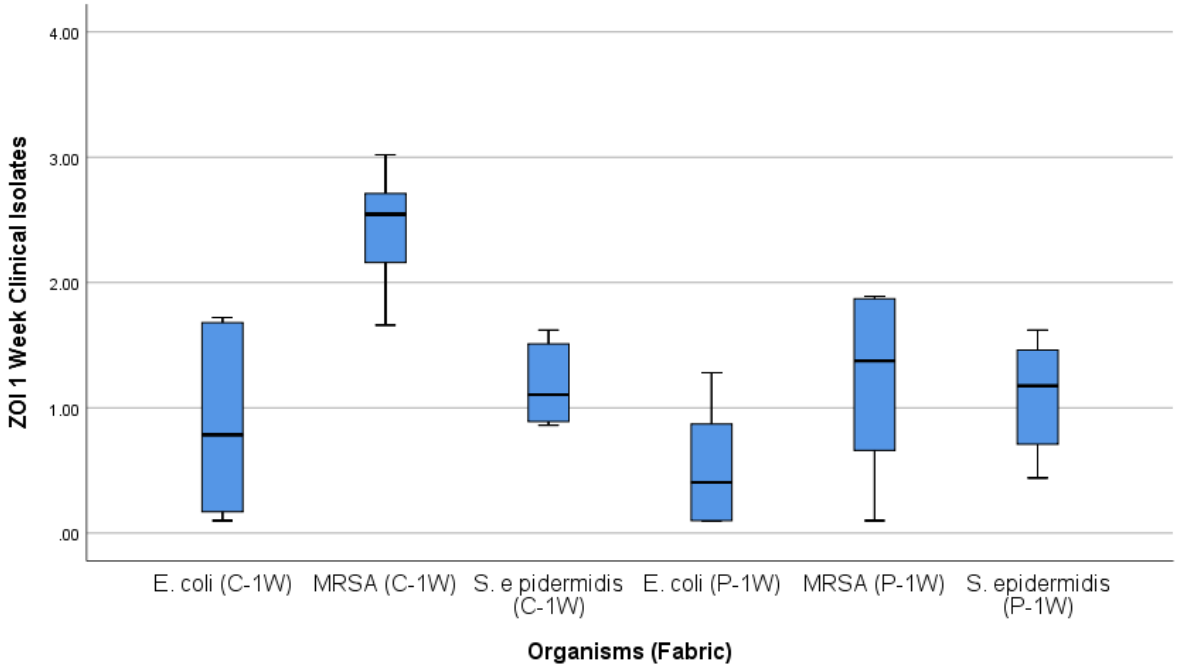
		Kolmogorov-Smirnov[a]			Shapiro-Wilk
	Organisms (Fabric)	Statistic	df	Sig.	Statistic
ZOI 1 Week Clinical Isolates	E. coli (C-1W)	.207	6	.200*	.881
	MRSA (C-1W)	.191	6	.200*	.960
	S. epidermidis (C-1W)	.193	6	.200*	.892
	E. coli (P-1W)	.232	6	.200*	.892
	MRSA (P-1W)	.173	6	.200*	.909
	S. epidermidis (P-1W)	.170	6	.200*	.956

Tests of Normality

		Shapiro-Wilk[a]	
	Organisms (Fabric)	df	Sig.
ZOI 1 Week Clinical Isolates	E. coli (C-1W)	6	.274
	MRSA (C-1W)	6	.818
	S. epidermidis (C-1W)	6	.331
	E. coli (P-1W)	6	.327
	MRSA (P-1W)	6	.429
	S. epidermidis (P-1W)	6	.785

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Descriptives

ZOI 1 Week Clinical Isolates

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound
E. coli (C-1W)	6	.8733	.70625	.28832	.1322
MRSA (C-1W)	6	2.4400	.48054	.19618	1.9357
S. epidermidis (C-1W)	6	1.1817	.32084	.13098	.8450
E. coli (P-1W)	6	.5267	.46543	.19001	.0382
MRSA (P-1W)	6	1.2117	.71205	.29069	.4644
S. epidermidis (P-1W)	6	1.0967	.44947	.18350	.6250
Total	36	1.2217	.78244	.13041	.9569

Descriptives

ZOI 1 Week Clinical Isolates

	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
E. coli (C-1W)	1.6145	.10	1.72
MRSA (C-1W)	2.9443	1.66	3.02

S. epidermidis (C-1W)	1.5184	.86	1.62
E. coli (P-1W)	1.0151	.10	1.28
MRSA (P-1W)	1.9589	.10	1.89
S. epidermidis (P-1W)	1.5684	.44	1.62
Total	1.4864	.10	3.02

Test of Homogeneity of Variances

		Levene Statistic	df1	df2
ZOI 1 Week Clinical Isolates	Based on Mean	1.090	5	30
	Based on Median	.906	5	30
	Based on Median and with adjusted df	.906	5	24.372
	Based on trimmed mean	1.063	5	30

Test of Homogeneity of Variances

		Sig.
ZOI 1 Week Clinical Isolates	Based on Mean	.386
	Based on Median	.490
	Based on Median and with adjusted df	.493
	Based on trimmed mean	.400

ANOVA

ZOI 1 Week Clinical Isolates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.636	5	2.527	8.624	.000
Within Groups	8.792	30	.293		
Total	21.428	35			

Multiple Comparisons

Dependent Variable: ZOI 1 Week Clinical Isolates

	(I) Organisms (Fabric)	(J) Organisms (Fabric)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Tukey HSD	E. coli (C-1W)	MRSA (C-1W)	-1.56667*	.31254	.000	-2.5173	-.6160

		S. epidermidis (C-1W)	-.30833	.31254	.919	-1.2590	.6423
		E. coli (P-1W)	.34667	.31254	.874	-.6040	1.2973
		MRSA (P-1W)	-.33833	.31254	.884	-1.2890	.6123
		S. epidermidis (P-1W)	-.22333	.31254	.979	-1.1740	.7273
	MRSA (C-1W)	E. coli (C-1W)	1.56667*	.31254	.000	.6160	2.5173
		S. epidermidis (C-1W)	1.25833*	.31254	.004	.3077	2.2090
		E. coli (P-1W)	1.91333*	.31254	.000	.9627	2.8640
		MRSA (P-1W)	1.22833*	.31254	.006	.2777	2.1790
		S. epidermidis (P-1W)	1.34333*	.31254	.002	.3927	2.2940
	S. epidermidis (C-1W)	E. coli (C-1W)	.30833	.31254	.919	-.6423	1.2590
		MRSA (C-1W)	-1.25833*	.31254	.004	-2.2090	-.3077
		E. coli (P-1W)	.65500	.31254	.316	-.2956	1.6056
		MRSA (P-1W)	-.03000	.31254	1.000	-.9806	.9206
		S. epidermidis (P-1W)	.08500	.31254	1.000	-.8656	1.0356
	E. coli (P-1W)	E. coli (C-1W)	-.34667	.31254	.874	-1.2973	.6040
		MRSA (C-1W)	-1.91333*	.31254	.000	-2.8640	-.9627
		S. epidermidis (C-1W)	-.65500	.31254	.316	-1.6056	.2956
		MRSA (P-1W)	-.68500	.31254	.271	-1.6356	.2656
		S. epidermidis (P-1W)	-.57000	.31254	.467	-1.5206	.3806
	MRSA (P-1W)	E. coli (C-1W)	.33833	.31254	.884	-.6123	1.2890
		MRSA (C-1W)	-1.22833*	.31254	.006	-2.1790	-.2777
		S. epidermidis (C-1W)	.03000	.31254	1.000	-.9206	.9806
		E. coli (P-1W)	.68500	.31254	.271	-.2656	1.6356
		S. epidermidis (P-1W)	.11500	.31254	.999	-.8356	1.0656
	S. epidermidis (P-1W)	E. coli (C-1W)	.22333	.31254	.979	-.7273	1.1740
		MRSA (C-1W)	-1.34333*	.31254	.002	-2.2940	-.3927
		S. epidermidis (C-1W)	.08500	.31254	1.000	-1.0356	.8656
		E. coli (P-1W)	.57000	.31254	.467	-.3806	1.5206
		MRSA (P-1W)	.11500	.31254	.999	-1.0656	.8356
Games-Howell	E. coli (C-1W)	MRSA (C-1W)	-1.56667*	.34874	.014	-2.8114	-.3219
		S. epidermidis (C-1W)	-.30833	.31668	.913	-1.5094	.8927
		E. coli (P-1W)	.34667	.34531	.905	-.8910	1.5843

	MRSA (P-1W)	-.33833	.40943	.956	-1.7604	1.0838
	S. epidermidis (P-1W)	-.22333	.34176	.983	-1.4542	1.0076
MRSA (C-1W)	E. coli (C-1W)	1.56667*	.34874	.014	.3219	2.8114
	S. epidermidis (C-1W)	1.25833*	.23589	.005	.4143	2.1023
	E. coli (P-1W)	1.91333*	.27311	.000	.9645	2.8621
	MRSA (P-1W)	1.22833	.35070	.055	-.0247	2.4814
	S. epidermidis (P-1W)	1.34333*	.26862	.005	.4095	2.2772
S. epidermidis (C-1W)	E. coli (C-1W)	.30833	.31668	.913	-.8927	1.5094
	MRSA (C-1W)	-1.25833*	.23589	.005	-2.1023	-.4143
	E. coli (P-1W)	.65500	.23078	.139	-.1673	1.4773
	MRSA (P-1W)	-.03000	.31884	1.000	-1.2408	1.1808
	S. epidermidis (P-1W)	.08500	.22545	.999	-.7149	.8849
E. coli (P-1W)	E. coli (C-1W)	-.34667	.34531	.905	-1.5843	.8910
	MRSA (C-1W)	-1.91333*	.27311	.000	-2.8621	-.9645
	S. epidermidis (C-1W)	.65500	.23078	.139	-1.4773	.1673
	MRSA (P-1W)	-.68500	.34729	.425	-1.9312	.5612
	S. epidermidis (P-1W)	.57000	.26415	.333	-1.4877	.3477
MRSA (P-1W)	E. coli (C-1W)	.33833	.40943	.956	-1.0838	1.7604
	MRSA (C-1W)	-1.22833	.35070	.055	-2.4814	.0247
	S. epidermidis (C-1W)	.03000	.31884	1.000	-1.1808	1.2408
	E. coli (P-1W)	.68500	.34729	.425	-.5612	1.9312
	S. epidermidis (P-1W)	.11500	.34376	.999	-1.1245	1.3545
S. epidermidis (P-1W)	E. coli (C-1W)	.22333	.34176	.983	-1.0076	1.4542
	MRSA (C-1W)	-1.34333*	.26862	.005	-2.2772	-.4095
	S. epidermidis (C-1W)	.08500	.22545	.999	-.8849	.7149
	E. coli (P-1W)	.57000	.26415	.333	-.3477	1.4877
	MRSA (P-1W)	-.11500	.34376	.999	-1.3545	1.1245

*. The mean difference is significant at the 0.05 level.

Table Appendix IV Modifications to Ross Y-Tube Olfactometer compared to WHO Olfactometer

	Ross Y-tube Olfactometer	WHO Y-tube Olfactometer
Material	Acrylic	Acrylic
Base leg length	50.0cm	45.72 cm
Holding port	21.0 cm	21.00 cm
Trapping port	15.4 cm	10.16 cm
Collar	20 cm	5.08 cm
Decision Chamber	YES	NO